Original article

Effect of creatine phosphate sodium on miRNA378, miRNA378* and calumenin mRNA in adriamycin-injured cardiomyocytes

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Abstract
Objective: To investigate the effect of creatine phosphate sodium (sodium phosphocreatine) on miRNA378, miRNA378* and calumenin mRNA in adriamycin-injured suckling mouse myocardium. Methods: The suckling mouse myocardium of primary culture were randomly divided into control group, adriamycin group and treatment group. To identify the suckling mouse myocardium, Smooth muscle actin-α (α-SMA) was monitored by immunohistochemical method. Cardiac function was evaluated by transthoracic echo-cardiography. The mRNA change of miRNA378, miRNA378* and calumenin mRNA were detected by quantitative real-time PCR. The expression of calumenin and GRP78 were identified by western blot. Results: Mitochondrial damage and vacuolization were found in adriamycin-induced suckling mouse myocardium compared with control group, while creatine phosphate sodium could reduce this phenomenon. Compared with the control group, the mRNA of miRNA378, miRNA378* and calumenin in adriamycin group was reduced, while creatine phosphate sodium could increase this phenomenon. The expression of calumenin and GRP78 were decreased after adriamycin treatment in suckling mouse myocardiums, creatine phosphate sodium increased the expression of calumenin and GRP78. Conclusion: The results of this experiment might be closely related to the effects of that creatine phosphate sodium reduced the pathological mechanism of suckling mouse myocardium with myocarditis caused by adriamycin.

Key words: adriamycin; creatine phosphate sodium; miRNA; calumenin; endoplasmic reticulum stress (ERS)

Introduction
As an anthracycline antibiotic, adriamycin is one of the most effective anti-neoplastic agent in recent years. High concentrations of adriamycin tend to cause severe toxicity to myocardium of which can turn into irreversible myocardial damage, even congestive heart-failure in the end [1]. The pathogenesis of adriamycin induced cardiomyopathy is not clear, it’s been considered that it has intimate connection with myocardial apoptosis [2-4]. Apoptosis plays an important role in development and in some physiological processes. For the past few years, we have learned that endoplasmic reticulum stress (ERS) pathway-mediated myocardial apoptosis is a new signal transduction pathway [5]. ER stress is a condition that is accelerated by the accumulation of unfolded or misfolded protein after a disturbance in the ER quality control system because of kinds of pathological and physiological occurrences [6]. Through researching ERS, it has been found the calumenin protein played an essential role in the alleviation of ERS in neonatal rat cardiomyocytes [7]. According to recent reports, the down regulation of calumenin in H9C2 cardiomyocytes might be associated with miRNA378 and miRNA378* [8]. Sodium phosphocreatine is a high efficient energy, which could provide energy in cardiomyocytes. The mechanism of sodium phosphocreatine has been reported, such as inhibit the permeability of cell...
membrane, change pore of myocardial to protect the mitochondria, even reduce apoptosis [9].

In this study, we investigated the potential mechanism of the protective effect of sodium phosphocreatine on adriamycin-induced cardiotoxicity injury. We suggested sodium phosphocreatine relieved adriamycin-induced cardiotoxicity by reduction ER-initiated apoptosis. This effect related with calumenin protein and miRNA378 and miRNA378*.

Materials and Methods

Materials

The 1-3d suckling mice were obtained from the Ji Lin university, license key: SCXK (Ji) 2011-0004; Super M-MLV reverse transcriptase from BioTeke Company, RNA simple Total RNA Kit from TIANGEN Company and Primers made from Sangon Biotech (Shanghai); Creatine phosphate sodium was purchased from the medicine of Ying Lian (Ji Lin, China), adramycin was purchased from the medicine of Wan Dong (ShenZhen, China).

Isolation and culture of myocardial cells of sucking mouse

The suckling mouse myocardium was put into the petridish and cleaned out several times with PBS. After the cleaning, it was cut into 1-3mm$^3$ pieces, then digested with 0.1% collagenase II solution and 0.1% trypsin at 37°C for 25 minutes. Then transferred to a 15 ml-centrifuge tube before centrifuged 1500 g×7 minutes, the supernatant was abandoned. Suspended the precipitate in PBS and homogenized by pipetting, centrifuged it for 5 minutes at 1 000 r/min, suspended the cells in medium, removed the residue with 200 screen mesh. Washed the cells with PBS twice and centrifuged for 10 minutes at 1 000 r/min, removed the supernatant fluid and obtained the precipitate. Added 1 ml suspended cells and transfused into the culture plate after cell-counting. Though the speed of fibroblasts adhering to the wall was really fast, a mass of fibroblasts could be cleaned out by the 2-hour differential velocity adherent technique, leaving the pure cardiomyocytes in the supernatant. The cells were placed in the culture plate over 24 hours. Adjusted the cell concentration to 5×10$^4$/ml, plated in 6-well tissue culture plates, incubated them in 5% CO$_2$ incubator for one night at 37°C. The cell density usually maintained at 70% appropriately.

Identify myocardial cells by immunohistochemical method

Immunohistochemical method was used to identify the myocardial cells. The cells were incubated in 0.1% Triton-100 and 3%Hydrogen peroxide. Then they were incubated with primary antibody and secondary antibody. Last using microscopic to exam.

Cardiomyocytes injury model by adriamycin induced

The suckling mouse myocardium were plated in 6-well tissue culture plates as the cell concentration of 5×10$^4$/ml and were incubated in 5% CO$_2$ incubator for one night at 37°C. Next day, the adriamycin infection group was added 3mg/L adriamycin and the creatine phosphate sodium group was added both 3 mg/L adriamycin and creatine phosphate sodium (10 mmol/L) each for 3 days, both groups were maintained at the temperature of 37°C [10].

Transmission electron microscopy analysis

The Cardiomyocytes were immersed in 4 % formalin in three groups. After dehydration in a graded concentration of alcohol, ultrathin sections of tissues were mounted on formvar-coated slot grids, stained with uranyl acetate, lead citrate, then examined with a JEOL 1200 electron microscope (JEOL Co., Tokyo, Japan).

Detected the expression of miRNA378, miRNA378* and calumenin mRNA of each group by Quantitative Real-time PCR

Informations of primers. CalumeninF: 5’GGT GAA GAC AGA GCG AGA AC 3’; CalumeninR: 5’ ATC TCC TCC TTG GTG AGC TTG 3’; RP78F: 5’ CAG CCA ACT GTA ACA ATC AAG 3’; RP78R: 5’ CTG TCA CTC GGA GAA TAC CA 3’; β-actinF: 5’ CTG TGC CCA TCT ACG AGG GCT AT 3’; β-actinR: 5’T TT GAT GTC ACG CAC GAT TTC C 3’.

Total RNA was extracted from the samples. RNA concentration Detection, reverse transcription and fluorescence quantitative PCR. A parallel experiment was made for each gene of every sample in 4 parallel holes and was repeated for 3 times. Test data with a wide deviation from the estimated value should be rejected, and the remaining test data should be averaged and taken as the required finally results. And the results should be analyzed by $2^{-\triangle\triangle CT}$ method.

Statistical analysis

All data were expressed as means±SE. Two-group
comparisons of the means were carried out by matched t-test using SPSS 11.5.

**Results**

The suckling mouse myocardium cells were isolated successfully by immunohistochemical

The suckling mouse myocardium cells were isolated by previous approaches. The morphology of isolated cells was nearly round at first. After 24 hour, the number of adherent cells was significantly increased and the morphology of adherent cells showed multiple angular or spindle (Fig.1A). Using alpha-smooth muscle actin (α-SMA), the suckling mouse myocardium cells were identified by immunohistochemical (Fig.1B).

**Ultrastructural remodelling of nucleus tractus solitarii**

Compared with control group, myocardial fiber dissolution, flake disappeared, part of cell nuclear membrane disappeared, cytoplasm concentration, mitochondrial damage and vacuolization were frequently found in adriamycin-injured suckling mouse myocardiocytes but minimal ultrastructural changes in similarly treated creatine phosphate sodium treated in adriamycin-injured suckling mouse myocardium. The structures of cardiac muscle fibers, mitochondria, nuclei and intercalated discs were not obviously abnormal in creatine phosphate sodium group (Fig.2).

**The expression of GRP78 was analyzed by quantitative real-time PCR**

Compared with the control group, the expression of GRP78 mRNA in creatine phosphate sodium was overtly increased. Compared with the adriamycin infection group, the expression of which in creatine phosphate sodium group was decreased (Fig.3).

**The expression of miRNA378 and miRNA378* were analyzed by quantitative real-time PCR**

Compared with the control group, the expression of miRNA378 and miRNA378* in adriamycin infection group was reduced. On the contrary, compared with the adriamycin infection group, the expression of which in creatine phosphate sodium group was increased. No changes were observed in the creatine phosphate sodium group (Fig.4).

**The expression of calumenin was analyzed by quantitative real-time PCR**

Compared with the control group, the expression of calumenin mRNA in adriamycin infection group was distinctly decreased. Compared with the adriamycin

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Tab. 1 Adriamycin-injured cardiomyocytes model preparation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (ng/μl)</th>
<th>260/280</th>
<th>Total quantities (ng)</th>
<th>Total volume (μl)</th>
<th>Sampling volume (μl)</th>
<th>Supplementary water capacity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244.5</td>
<td>1.82</td>
<td>1000</td>
<td>10.5</td>
<td>4.1</td>
<td>6.4</td>
</tr>
<tr>
<td>ADR</td>
<td>159.5</td>
<td>1.93</td>
<td>1000</td>
<td>10.5</td>
<td>6.1</td>
<td>4.4</td>
</tr>
<tr>
<td>CPS</td>
<td>305.8</td>
<td>1.90</td>
<td>1000</td>
<td>10.5</td>
<td>3.3</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Experimental data of each sample. Sample1: The control group. Sample2: The ADR group. Sample3: The CPS group. ADR, adriamycin group; CPS, creative phosphate sodium group.
infection group, the expression of which in creatine phosphate sodium group was visibly increased (Fig. 5).

Discussion

In this present research, we prove the underlying mechanisms of sodium phosphocreatine against adriamycin induced cardiotoxicity. This mechanism related with ER stress by calumenin protein and miRNA378 and miRNA378*.

Adriamycin induced cardiotoxicity was involved with myocardial apoptosis has been reported [11]. However, the mechanism of sodium phosphocreatine related with apoptosis [9]. ER stress induced apoptosis has been studied [12]. Endoplasmic reticulum (ER) has been linked to many diseases, including cardiotoxicity. The accumulation of misfolded protein disrupts ER and leads to the activation of the classic coping mechanism termed the unfolded protein response [13]. ER alleviated and relieved itself by several ways such as upregulating the expression of GRP78 inhibiting protein synthesis, accelerating the misfolding protein and degrading the unfolding protein. To investigate whether sodium phosphocreatine would relieve ER damage, we examined grp78 gene by real-time PCR. Form the result, we demonstrated a dose-related decrease in the mRNA expression of grp78 after treating with sodium phosphocreatine. The result suggested that ER stress can be relieved in adriamycin-induced cardiotoxicity after treating with sodium phosphocreatin.

Recent studies demonstrated that miRNA378 has the ability to regulate the activation of cardiac fibroblasts by indirect modulation of TGFβ1 synthesis and release from cardiomyocytes [14]. The experimental evidence showed that the inhibition of miRNA378 induced the cardiac fibroblasts of the suckling mouse [15]. The higher the degree of heart failure, the lower the expression of miRNA378 and miRNA378* in the severe heart failure patients. Luckily, the expression of
miRNA378 and miRNA378* was increased significantly with the improvement of the patients’ cardiac function [16]. The down regulation of calumenin in h9c2 cardiomyocytes might be associated with miRNA378 and miRNA378* [17]. Calumenin is a CREC family Ca2+-binding protein with multiple EF-hand motifs which is located in the endo/sarcoplasmic reticulum of mammalian cardiomyocytes [18]. Calumenin plays an essential role in the alleviation of ERS in neonatal rat cardiomyocytes. At the same time, calumenin reduced ER-initiated apoptosis in neonatal rat ventricular cardiomyocytes [19]. In our study, we tested the expression of miRNA378, miRNA378* and calumenin by real-time PCR. The expression of miRNA378, miRNA378* and calumenin were down-regulated after treating with adriamycin in cardiomyocytes. Sodium phosphocreatine up-regulated this phenomenon.

The cardiomyocytes injury caused by adriamycin reduced the expression of miRNA378, miRNA378* and calumenin mRNA and increased the expression of GRP78 mRNA. Sodium phosphocreatine activated the phenomenon.

The experimental results confirmed that parts of our scientific hypothesis was established. Sodium phosphocreatine did relieve the ERS by increasing the expression of mRNA of miRNA378, miRNA378* and calumenin.

References
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