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Study on the effect of doxorubicin on expressions of genes encoding myocardial sarcoplasmic reticulum Ca^{2+} transport proteins and the effect of taurine on myocardial protection in rabbits

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Abstract: To investigate the effect of doxorubicin(DOX) on gene expression of the myocardial sarcoplasmic reticulum (SR) Ca^{2+} transport proteins and the mechanism of taurine(Tau) protecting cardiac muscle cells, 9 rabbits were injected with DOX, 8 rabbits with DOX and Tau, and 9 rabbits with normal saline. Cardiac function, concentration of calcium in cardiomyocytes (Myo$[Ca^{2+}]_i$), activity of SR Ca^{2+}-ATPase (SERCA2a), level of SERCA2a mRNA and Ca^{2+} released channels(RYR2)mRNA were detected. The left ventricle tissues were observed by electron microscopy. The results showed that cardiac index, left ventricular systolic pressure, activity of SR Ca^{2+}-ATPase and level of SERCA2a mRNA decreased, while Myo$[Ca^{2+}]_i$ increased in DOX-treated rabbits. DOX could not affect the level of RYR2 mRNA. Tau intervention could alleviate the increase of left ventricular diastolic pressure, Myo$[Ca^{2+}]_i$ and the decrease of SERCA2a mRNA induced by doxorubicin. The results suggested that downregulation of SERCA2a gene expression was an important mechanism of DOX-induced cardiomyopathy and that Tau could partially improve the heart function by reducing calcium overload and alleviating downregulation of SERCA2a mRNA.

Key words: Doxorubicin, Ca^{2+}-ATPase, Ryanodine receptor, Taurine, Gene expression
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INTRODUCTION

Doxorubicin(DOX) is a kind of anthracyclines antibiotics whose antineoplastic spectrum is broad and effect is strong. It has gained widespread use in the treatment of childhood leukemia and solid tumors. However, its clinical use is limited by its cardiotoxicity. The calcium overload in cardiomyocytes could be closely correlated to doxorubicin cardiotoxicity(Maeda et al., 1998). It was known that intracellular free Ca^{2+} concentration was mainly regulated by sarcoplasmic reticulum(SR) Ca^{2+} transport proteins, such as the Ca^{2+} released channels (Rynodine receptors, RYR2) and Ca^{2+}-ATPase (SERCA2a) on the myocardial sarcoplasmic reticulum membrane. It was found that abnormality of RYR2 and SERCA2a genes expressions existed in many heart diseases(Huang et al., 1999). Recent evidences suggested that Taurine (Tau) could regulate Ca^{2+} concentration in cardiomyocytes and inhibit calcium overload(Shu et al., 1997).

Our objective was to assess the effects of chronic doxorubicin administration and oral intake of Tau on Ca^{2+} transport proteins so as to uncover the mechanism of doxorubicin-induced cardiomyopathy as well as the mechanism of how Tau prevents hearts from the developing cardiomyopathy.

METHODS

Animal models

Twenty-six New Zealand white 3 - 4 months old male and female rabbits (provided by the Experimental Animals Center in Zhejiang) were randomly divided into three groups. DOX group: Nine rabbits (1.6 - 1.95kg, mean 1.74 ± 0.12 kg) were treated with intravenously injected with doxorubicin, 2 mg/kg, once a week for 8 weeks. Doxorubicin was diluted to 1 mg/kg with saline. DOX + Tau group: Eight rabbits(1.55 - 2.10 kg, mean 1.71 ± 0.12 kg) were treated just like

the DOX group but were also treated with oral intake of Tau (100 mg/kg, once a day for 6 weeks) after 5 weeks of injecting DOX. Control group (NS group): Nine rabbits (1.6 – 2.05 kg, mean 1.79 ± 0.14 kg) were treated with injection of normal saline(NS), 2 ml/kg, once a week for 8 weeks. Studies were conducted on the 4th week after the final injection of doxorubicin to avoid any acute phase effects of doxorubicin. Age and weight were not significantly different among these three groups.

Methods

After their body weights were measured, the rabbits were anesthetized with sodium pentobarbital (25 – 50 mg/kg).

1. Cardiac index (CI) analyses: A probe of the Doppler flow meter (MARK-600, Pulsed Dopller Flow/Dimension System. ATL Co, USA. Diameter of probe: 6 mm. Frequency: 5MHz) was attached to the inferior xiphoid process to evaluate the flow velocity of the descending aorta. The diameter of the descending aorta was determined after right thoracotomy. The heart rate and blood flow of the descending aorta per time unit were calculated, the latter represented cardiac output, from which CI could be calculated according to cardiac output.

2. Blood pressure(Bp), left ventricular systolic pressure(LVSP) and left ventricular diastolic pressure(LVDP) analyses: The left carotid artery of each rabbit was cannulated with a 2F polyethylene catheter connected to a cardiaopulmonary monitor by pressure transducer(Sirecust 732 type, Siemens, Corp., Germany) for calculation of Bp; after which the catheter was cannulated into the left ventricular for calculation of LVSP and LVDP.

3. Ventricle preparation: Cold and high potassium Tyrode's solution (12 mmol/L KCl, 138 mmol/L NaCl, 1 mmol/L $MgCl_2$, 10 mmol/L Glucose, 0.33 mmol/L NaH_2PO_4, 10 mmol/L HEPES) was perfused at the aorta root till cardiac arrest. The heart was then excised and the atria was trimmed away. The ventricular was rinsed in cold saline, blotted on sterile paper towels. The left ventricular tissue was transversely sectioned. A piece of left ventricular tissue(0.6 cm × 0.6 cm × 0.3 cm) was promptly used to calculate Myo$[Ca^{2+}]_i$, another piece! was put into 10% glutaraldehyde so as to be used for observation under electron microscope; another pieces was immediately frozen in liquid nitrogen.

4. Myo$[Ca^{2+}]_i$ analyses: Free Ca^{2+} in cardiac cells was detected by the modified method of Beuckelmann et al. (1992). The cardiac cells were separated by type I collagenase digestion and labeled by fura-2 ale foxylmethyl ester (fura-2/AM).

5. SRCa^{2+}-ATPase analyses: Firstly, SR was prepared by the differential centrifugation method of Jones et al. (1979). Secondly, the concentration of SR protein was measured by Coomassie brilliant blue staining (Bradford, 1976). Finally, the activity of SR Ca^{2+}-ATPase was determined by the inorganic phosphorus method(Suen, 1996).

6. SERCA2a mRNA and RYR2 mRNA analyses: SERCA2a and RYR2 mRNA levels were detected by RT-PCR technique and semi-quantitative method (RT-PCR kit produced by Boehringer Mannheim, Germany). The primers for the amplification of cardiac RYR, SERCA2a, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were designed from published rabbit sequences (MacLennan et al., 1985; Otsu et al., 1990; Applequist et al., 1995). The primers were synthesized by Sangon Bio-tech Inc. Shanghai.

For SERCA2a (477bp): forward, 5' TTGCATTGCAGTCTGGATCA 3'; reverse, 5' TCCAAAGCAGAGTCATTACA 3'. For RYR2 (487bp): forward, 5' GGAAATCCATTCTGAATT-CTC 3'; reverse, 5' GCAGTCACAAACGGCTCGGTG 3'. For GAPDH (726bp): forward, 5' ATTCATTGACCTCCACTACATGGT 3'; reverse, 5' CTCGGTGTAGCCCAGGATGCCCTT 3'.

The GAPDH (internal control) primers together with the RYR2 primers or SERCA2a primers were put into a tube for amplification of GAPDH and RYR2 or GAPDH and SERCA2a. The PCR products were subjected to 2% polyacrylamide gel electrophoresis, and then associated densities were measured by scan with IS-1000-digital imaging system (Alpha-innotech Inc). The relative density with RYR2 or SERCA2a PCR products in each sample was calculated by dividing the density associated with RYR2 or SERCA2a PCR products by that associated with

the GAPDH gene product.

Statistical analysis

Data were expressed as means ± s. All data were given in logarithmic units. Differences in the logarithms of the mean values of data from three groups rabbits were assessed using the variance analysis. Linear regression analysis was used to assess the possible correlation. Statistics were done with SPSS 7.0 software. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Comparison of hemodynamics

Table 1 shows that the heart rates were not significantly different between DOX-treated rabbits and the controls. But the cardiac index and BP and LVSP of the DOX-treated rabbits decreased dramatically. LVDP of the DOX group was significantly higher than that of the control group. There was no statistical difference between CI of Tau intervention group and that of DOX group. In addition, Tau intervention could not alleviate decreasing of BP, LVSP induced by DOX. There were no differences between these two indexes of the Tau-intervened group and those of the DOX group. LVDP of DOX + Tau group rabbits was significantly lower than that of DOX group rabbits, but was not different from that of the control ones.

Table 1 Comparison of hemodynamics of three rabbits groups(x ± s)

	n	HR	CI	BP	LVSP	LVDP
NS	9	248 ± 16	1.70 ± 0.40**	115 ± 12/100 ± 13**	106.9 ± 12.6**	1.1 ± 0.6**
DOX	9	237 ± 21	1.24 ± 0.39	84 ± 8/71 ± 8	80.2 ± 12.7	7.2 ± 5.7▲
DOX + Tau	8	248 ± 25	1.65 ± 0.58	89 ± 13/77 ± 12*	82.3 ± 18.3*	3.1 ± 4.8
F		0.850	3.469	15.092	4.225	4.236
P		> 0.05	< 0.05	< 0.01	< 0.05	< 0.05

** Comparison between DOX group and NS group, $P < 0.05$; * Comparison between DOX + Tau group and NS group, $P < 0.05$; ▲ Comparison between DOX group and DOX + Tau, $P < 0.05$.

Comparison of Myo$[Ca^{2+}]_i$ and SR Ca^{2+}-ATPase activity

Table 2 shows that concentration of free Ca^{2+} in the cardiac cells of DOX group was dramatically higher than that of control groups ($P < 0.05$). Whereas, SR Ca^{2+}-ATPase activity of DOX groups was significantly lower than that of control groups ($P < 0.05$). The level of Myo $[Ca^{2+}]_i$ of DOX + Tau group was significantly lower than that of DOX group ($P < 0.05$), and not significantly different from that of NS group ($P > 0.05$). There was no significant difference between the SR Ca^{2+}-ATPase activity of DOX + Tau group and that of DOX group ($P > 0.05$). Linear regression analysis indicated that Myo $[Ca^{2+}]_i$ and CI were negatively correlated($r = -0.727$, $P < 0.05$); that SR Ca^{2+}-ATPase activity and CI were positively correlated ($r = 0.568$, $P < 0.05$); and that SR Ca^{2+}-ATPase activity and Myo$[Ca^{2+}]_i$ were negatively correlated($r = -0.629$, $P < 0.05$).

Comparison of the gene expression of SERCA2a and RYR2

As shown in Table 3, Fig. 1 and Fig. 2. SERCA2a mRNA level decreased significantly ($P < 0.05$) in DOX group rabbits. SERCA2a mRNA level of DOX + Tau group was significantly higher than that of DOX group and lower than that of NS group. RYR2 mRNA level of DOX + Tau group was not significantly different from that of DOX group and that of the control group respectively ($P > 0.05$).

Table 2 Comparison of Myo$[Ca^{2+}]_i$, SR Ca^{2+}-ATPase activity of three rabbit groups($\chi \pm s$)

	n	Myo$[Ca^{2+}]_i$	SRCa^{2+}-ATPase activity
NS	9	$16.57 \pm 3.81^{**}$	$76.64 \pm 11.65^{**}$
DOX	9	$53.48 \pm 33.40^{▲}$	43.32 ± 9.84
DOX + Tau	8	26.20 ± 9.54	$43.92 \pm 13.02^{*}$
F		7.821	14.872
P		< 0.01	< 0.01

** Comparison between DOX group and NS group, $P < 0.05$; * Comparison between DOX + Tau group and NS group, $P < 0.05$; ▲ Comparison between DOX group and DOX + Tau, $P < 0.05$.

Table 3 Comparison of level of SERCA2a mRNA and RYR2 mRNA of three rabbit groups($\chi \pm s$)

	n	SERCA2a mRNA	RYR2 mRNA
NS	5	$0.52 \pm 0.12^{**}$	0.54 ± 0.14
DOX	5	$0.28 \pm 0.06^{▲}$	0.41 ± 0.03
DOX + Tau	5	$0.39 \pm 0.06^{*}$	0.47 ± 0.08
F		10.905	3.455
P		< 0.01	> 0.05

** Comparison between DOX group and NS group, $P < 0.05$; * Comparison between DOX + Tau group and NS group, $P < 0.05$; ▲ Comparison between DOX group and DOX + Tau, $P < 0.05$.

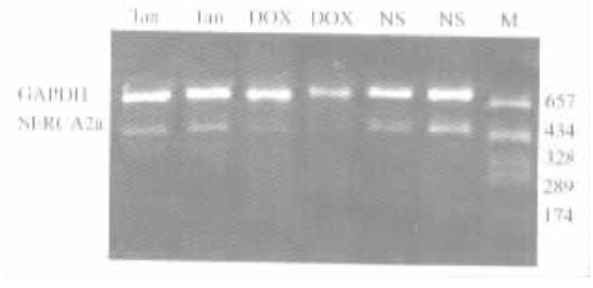

Fig.1 RT-PCR showing the levels of mRNA encoding SR SERCA2a in DOX-treated rabbits, DOX + Tau group rabbits and control rabbits

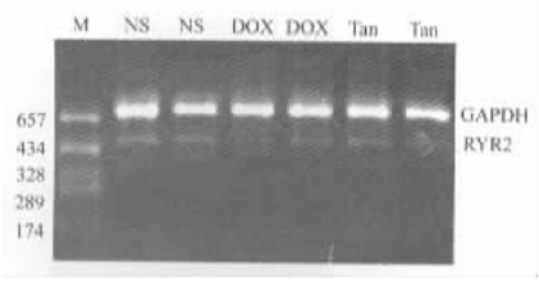

Fig.2 RT-PCR showing the levels of mRNA encoding SR RYR2 in DOX-treated rabbits, DOX + Tau group rabbits and control rabbits

Observation of the histological sections of the left ventricular myocardium under electron microscope.

As shown in Fig.3, myofilaments of the control group arranged regularly. Z and M line, light and dim band were clear. On the contrary, myofilaments of DOX group were loose and broken, cytoplasm lysed. Z and M line, light and dim band were indistinct, SR expanded, ribose bodies had come off, mitochondria were obviously hypertrophy and ranges became thicker in DOX group. Tau could alleviate DOX-induced injuries although loose and broken myofilaments, cytoplasm lyses, indistinct light and dim band, and Z line, still existed. Expansion of SR and hypertrophy of mitochondria of the Tau group were better than those of the DOX group although the Tau group failed to recover to the normal level.

DISCUSSION

Previous studies showed that DOX could cause chronic cardiotoxicity. When cumulative doses exceed 500 mg/m^2 of the body surface area, the incidence of congestive heart failure, known as doxorubicin cardiomyopathy, increases rapidly(Shan et al., 1996).

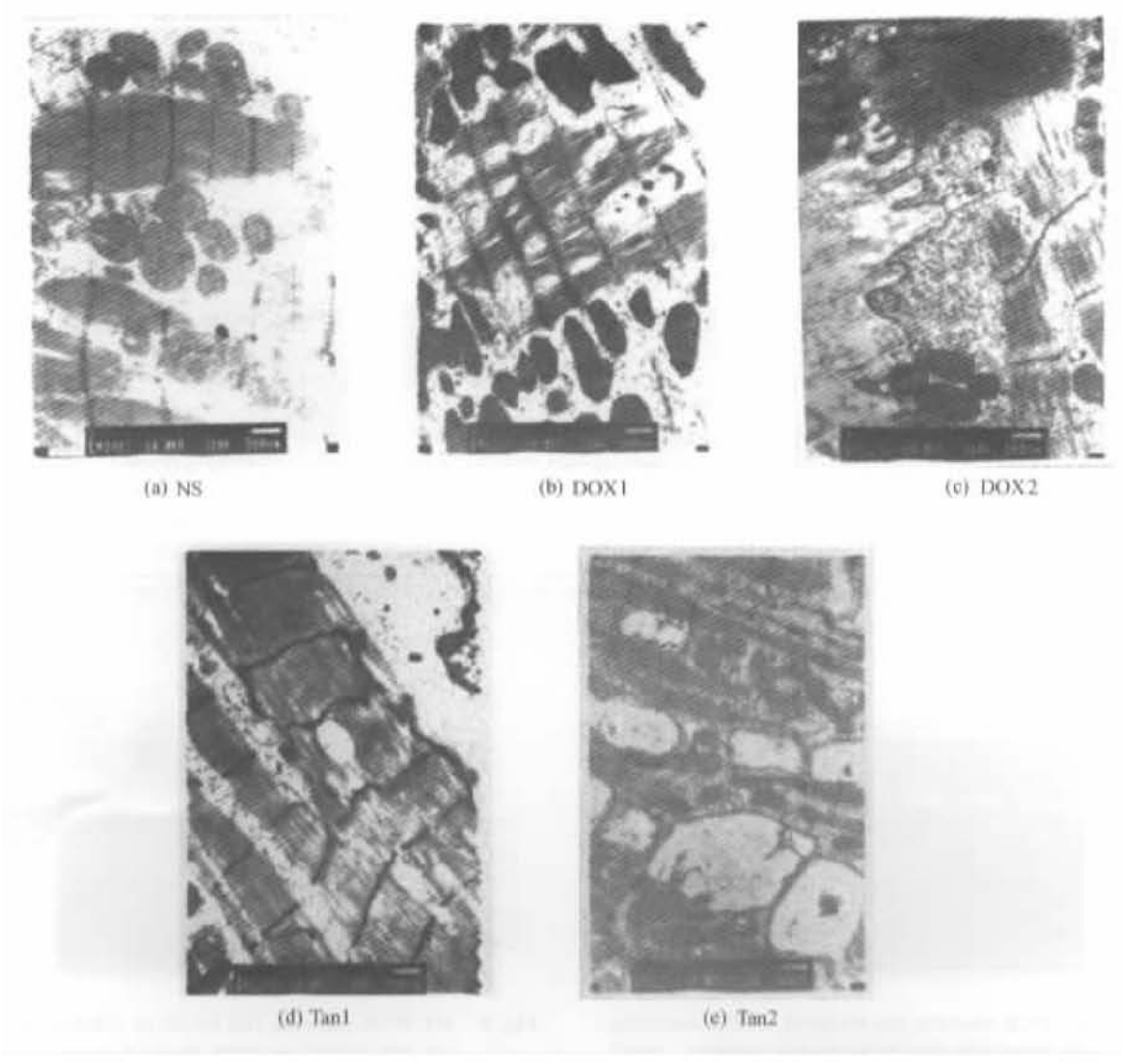

Fig.3 Scope (×10K) (a)NS: Myofilaments of NS group arranged regularly. Z and M line, light and dim band were clear; (b) DOX1: show: Myofilaments of DOX group were loose and broken, cytoplasm lysed. Z and M lines were indistinct, so were light and dim band. Mitochondria were obviously hypertrophied; (c) DOX2: show: SR of DOX group expanded. Some ribose bodies had come off ; and abnormal filar substances could be seen; (d) Taul: Tau could alleviate DOX-induced injuries although loose and broken myofilaments, cytoplasm lyses, indistinct light and dim band and Z line still existed; (e) Tau2: Expansion of SR and hypertrophy of mitochondria of the Tau group were better than those of the DOX group although the Tau group failed to recover to the normal level

To further investigate the pathogenesis of chronic DOX-induced cardiotoxicity in children, we investigated the long-term effects of DOX treatment on hearts in young rabbits, treated with accumulative doses of 16 mg/kg (which in them approximated to 320 mg/m^2) to mimic the cumulative administration of DOX during anti-cancer chemotherapy in children.

The present studies showed that CI, LVSP, SR Ca^{2+}-ATPase activity and the level of SR SERCA2a mRNA in DOX group rabbits were significantly reduced; and that the LVDP, Myo $[Ca^{2+}]_i$ of DOX group rabbits were significantly higher than that of the control group. Pathologi-

cal injuries of cardiac tissues existed obviously. The relationship between Myo$[Ca^{2+}]_i$ and CI and between Myo$[Ca^{2+}]_i$ and SR Ca^{2+}-ATPase activity were negatively correlated. On the contrary, the relationship between SR Ca^{2+}-ATPase activity and CI was positively correlated. The results indicated that DOX could induce the calcium overload in cardiomyocytes by inhibiting the activity of SR Ca^{2+}-ATPase, which was closely related to heart failure. The histological injuries of the left ventricular that were shown in our studies may be partially related to the cardiomyocyte autolysis caused by the calcium overload induced release of the enzymes in cells. The down-regulation of gene expression of SERCA2a, which is a protein transporting Ca^{2+} into SR, indicated that the reduction of SR Ca^{2+}-ATPase activity was partially caused by the depressed transcription of SERCA2a mRNA. We also found the mRNA expression levels of the major SR proteins used to Ca^{2+} release (RYR2) were not reduced in the hearts of the DOX-treated rabbits. It might indicate that the injuries of SR uptaking Ca^{2+} function occurred prior to those of SR releasing Ca^{2+} function in DOX-induced cardiomyopathy.

Tau is a β-sulfonic amino acid transformed from cysteine. It is found at very high concentrations in excitable tissues, particularly in the heart where it constitutes more than 50% of the total free amino acid pool(Timbrel, 1995). Previous study showed that taurine was of benefit in patients resistant to therapy with digitalis and diuretics (Huxtable, 1992). Dogs with dilated cardiomyopathy (DCM) are taurine-deficient. The myocardial functions of dogs with DCM were improved after they were treated with taurine for 4 months. During which, each dog was weaned off its cardiovascular drugs. Although the myocardial function did not return to normal in most of the dogs, it improved enough to allow discontinuation of cardiovascular drug therapy and to maintain a normal quality of life for months or years (Kittleson et al., 1997). The present studies showed Tau intervention could alleviate pathological injuries of the ventricular tissues induced by DOX. Tau intervention could increase CI of the DOX group from 1.24 ± 0.39 L/ml·m^2 to 1.65 ± 0.58 L/ml·m^2; CI of DOX + Tau group rabbits was 25% higher than that of DOX group rabbits. LVDP of DOX + Tau group rabbits was significantly lower than that of DOX group rabbits, and was not different from that of control ones. In addition, Tau intervention could not alleviate DOX induced decrease of BP, LVSP. There were no differences between these two indexes of the Tau-intervened group and those of the DOX group. It suggested that Tau intervention partially protects the heart against DOX-induced cardiotoxicity, but fails to return the heart function to normal. Because in our studies Tau was given only after 5 weeks injection of DOX, the heart tissue might have already been impaired during that time. Accordingly, we considered that intake of Tau at this time could only ameliorate deterioration but could not reverse the heart tissue injuries which had existed. At present it can't be affirmed which way is more effective, oral intake of Tau, before or simultaneously with DOX injection.

Recent evidence suggested the effect of Tau on the heart was positively correlated to calcium in cells. Tau could modulate the Ca^{2+} transport of cardiomyocytes and/or regulate the sensitivity of the target organs to Ca^{2+}, and preserve the Ca^{2+} homeostasis in cardiomyocytes, and afford protection against Ca^{2+}-overload in cardiomyocytes(Shu et al., 1997). Ji et al.(1997) found Tau could improve SR Ca^{2+}-ATPase activity and enhance the strength of [^{3}H]ryanodine binding to cardiac SR in septic rats with shock. It was suggested that Tau could improve the Ca^{2+} uptake and enhance the SR of animals suffering from acute heart injuries. Whereas, Tau added to incubation fluid failed to increase the Ca^{2+} uptake and enhance [^{3}H] ryanodine binding to the SR isolated from normal rats. It indicated that the mechanism of Tau acting on SR was still unclear. There is no report up to now if Tau affects SR Ca^{2+}-ATPase activity and the gene expressions of SR Ca^{2+} transport proteins in chronic DOX-induced cardiotoxicity. This study revealed that Tau intervention could reduce the increase of Myo$[Ca^{2+}]_i$ and the decrease of SERCA2a mRNA induced by DOX. The results showed that Tau intervention could improve the heart function by inhibiting DOX-induced Ca^{2+} overload and regulating expression of SERCA2a. Labudova et al.(1996) found that Tau could upregulate the expression of cation-transport AT-

Pase in rat heart. The upregulation of SERCA2a gene expression induced by Tau may be a heart protecting mechanism of Tau . We did not find Tau could alleviate the decrease of $SRCa^{2+}$-ATPase activity induced by DOX. Accordingly, we assumed that Tau could modulate the SERCA2a gene expression by affecting transcription of SERCA2a gene. The change of $SRCa^{2+}$-ATPase activity and that of SERCA2a expression induced by Tau intervention were inconsistent in this study. We assumed possible reasons were that this method of Tau intervention could not neutralize some factors that inhibited $SRCa^{2+}$-ATPase activity and/or rarely improved SERCA2a transcription but did not affect the translation of SERCA2a mRNA. In addition, the present study showed that RYR2 gene expression did not change after DOX injection and Tau intervention respectively. The results might indicate that the effect of DOX on SERCA2a gene expression was earlier than that of DOX on RYR2 gene expression and that Tau could not affect RYR2 gene transcription when the level of SR RYR2 mRNA was basically normal. It is necessary to study further whether Tau could affect RYR2 gene expression chronically injuried by DOX. Previous studies showed that Tau could modulate Ca^{2+} homeostasis by various ways. Tau could act on not only SR membrane but also the L-type Ca^{2+} channel, T-type Ca^{2+} channel, Ca^{2+}-ATPase and Na^{+}-Ca^{2+} exchanger on the plasma membrane, and so on (Shu et al., 1997). This study revealed that although $SRCa^{2+}$-ATPase activity had not been increased, Ca^{2+} overload in cells had been ameliorated after Tau intervention. Accordingly, the mechanism of Tau reducing Ca^{2+} overload in this study may be that Tau affected the Ca^{2+} transport of plasma membrane. It is necessary to study further whether extending the period of Tau treatment or increased dosage of Tau intake, could enhance the activity of SR-Ca^{2+}-ATPase.

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