

Evaluation of biomarkers for doxorubicin-induced cardiac injury in rats

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Abstract. Drug-induced cardiotoxicity is a leading cause of failure in drug development and predicting its occurrence in non-clinical studies is the primary preventive measure. The present study aimed to evaluate the changes in biomarkers during acute and chronic myocardial injury induced by doxorubicin (DOX) in rats. A rat model of acute myocardial injury was established through a single-dose, intraperitoneal injection of DOX (40 mg/kg), the changes in biomarkers were measured at 2, 4, 8 and 24 h after administration, following DOX administration, creatine kinase (CK) and fatty acid-binding protein 3 (FABP3) levels increased between 8 and 24 h, whereas cardiac troponin I (cTnI) peaked at 8 h. To establish a chronic myocardial injury model, rats received 1, 2 or 3 mg/kg DOX weekly by caudal vein injection for 2, 4, 6 or 7 weeks, the changes in biomarkers were detected at 2, 4, 6 and 8 weeks, the results showed that cTnI increased significantly after 2 and 8 weeks of administration. A significant increase in FABP3 and microRNA (miR)-146b levels was observed after 8 weeks of administration. Receiver operating characteristic curve and correlation analysis showed that cTnI and miR-146b had relatively high predictive values for chronic myocardial injury (area under the curve, 0.83 and 0.71, respectively) and were closely correlated with myocardial damage. These data suggested that CK, cTnI and FABP3 were relatively sensitive to DOX-induced acute myocardial injury, whereas cTnI and miR-146b were relatively sensitive to DOX-induced chronic myocardial injury.

Introduction

Drug-induced cardiotoxicity is a primary cause of failure in drug development, with approximately one-third of cases of drug development failure attributed to safety problems associated with cardiovascular toxicity (1-4). Failure in the development of candidate drugs is an economic loss for pharmaceutical companies, whereas side effects and toxicity threaten the health of patients (3,5). Therefore, successfully predicting drug-induced cardiotoxicity in non-clinical studies is important in decreasing drug development failure and clinical adverse reactions.

Current non-clinical safety evaluation methods for drug-induced myocardial injury in experimental animals include pathomorphological, clinicopathological and safety pharmacological examination (such as assessment of blood pressure, heart rate and electrocardiography) (S6 (R1): Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, S7A: Safety Pharmacology Studies for Human Pharmaceuticals) (6,7). In non-clinical safety studies, cardiac pathomorphological examinations are the gold standard for evaluating myocardial injury in experimental animals (8,9). However, this evaluation system is insufficient because certain drugs are still eliminated owing to cardiotoxicity in clinical trials, while others are withdrawn from the market owing to cardiotoxicity in clinical application. Thus, predicting the occurrence of drug-induced cardiotoxicity at the early stage of drug development is important. Furthermore, the lack of sensitive and specific markers prevents prediction at an early stage of the injury, which can lead to irreversible myocardial damage; thus, drug-induced chronic myocardial injury remains difficult to evaluate. More sensitive and specific evaluation methods are needed, among which use of predictive biomarkers is most valuable to predict cardiotoxicity in non-clinical safety evaluations and to monitor clinical drug-induced myocardial injury (10).

Traditional cardiotoxicity markers, such as CK and lactate dehydrogenase (LDH), have poor specificity (11). cTn is a sensitive and specific indicator of myocardial necrosis, and the increase in cTn levels is associated with the degree of myocardial injury (12). Therefore, cTn (cTnI and cTnT) is considered a good indicator for myocardial injury in experimental animals in non-clinical safety evaluations (12). However, there are

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limitations to the use of cTn. First, the detection methods have been developed and optimized for humans rather than for animals (13). Second, cTn is cleared rapidly in rat plasma (14) and blood collection time points are restricted, blood must be collected before cTn is cleared. Third, the cTn complex comprises three subunits that bind to thin myofilaments of striated muscles, troponin I, T, and C (15). A previous study suggested that cTnI and cTnT have strong predictive power for myocardial necrosis and are markers of structural damage, rather than early myocardial injury (16). FABP3 is a low molecular weight protein involved in lipid transport, storage, signal transduction, oxidation and transcriptional regulation (17), FABP3 are abundant in myocyte cytoplasm and rapidly released with cell injury (18), so it is another early biomarkers for assessing cardiomyocyte degeneration and necrosis.

DOX is an anthracycline antibiotic and its toxic effect on cardiomyocytes is an important research area (19). Although the specific mechanism remains unclear, DOX toxicity primarily involves oxidative stress, DNA/RNA damage, endoplasmic reticulum-mediated apoptosis and disturbance of calcium homeostasis (20). Apoptosis or necrosis of cardiomyocytes causes a release of enzymes and structural proteins, such as CK or cTn, into the blood. Over production of reactive oxygen species (ROS) occurs during oxidative stress; ROS activate a variety of signaling kinases and transcription factors, such as MAPK and NF- κ B (21), which may be responsible for changes in miRNA levels.

MicroRNAs (miRNAs) are short non-coding RNAs (~22 nucleotides long) that are relatively highly conserved (22). Most miRNAs in circulation originate from blood and endothelial cells and are found in the plasma of humans. The levels of cardio-specific miRNAs are low and an increase suggests that myocardial injury has occurred, such as miR-29a (23). miRNAs serve important roles in cardiac function and cardiovascular disease (24) and have emerged as key regulators of cardiac injury (25). miR-31 has been reported to participate in cardiac disorders, such as ischaemic heart diseases and arrhythmia (26). Yang *et al* (27) found that abnormal expression of miRNA (miR-499) leads to irreversible myocardial damage; that study also reported that DOX significantly increases the expression of miR-140-5p in rat heart tissue, leading to increased myocardial oxidative damage (28). miR-208a is a cardio-specific miRNA, the level of which increase significantly following repeated administration of isoprenaline (29). miR-208a silencing alleviates DOX-induced myocardial apoptosis in Balb/c mice (30). These reports demonstrate that miRNAs are potential biomarkers for cardiotoxicity.

The present study aimed to analyze changes in the levels of seven biomarkers [CK, LDH, cTnI, cTnT, FABP3, miR-146b, and miR-208a] in DOX-induced rat models of acute and chronic myocardial injury. The change in expression patterns of these markers and their predictive value for myocardial injury, as well as the correlation between cardiotoxicity risk and biomarker levels were also analyzed.

Materials and methods

Animals and experimental design. Male 7-week-old Sprague-Dawley rats (weight, 174–213 g) were purchased from

Beijing Vital River Laboratory Animal Technology Co., Ltd. They were maintained at 2 or 3 rats/cage, with unrestricted standard diet and sterilized water, temperature of 21.7–24.4°C, relative humidity of 44.1–72.2% and a 12/12-h light/dark cycle.

In the acute myocardial injury model, 29 rats were randomly divided into control group and DOX-treated group (Table I). The animals in the treated group were intraperitoneally injected with 40 mg/kg (10 ml/kg) DOX (lot no. H44024359; Shenzhen Main Luck Pharmaceuticals, Inc.); whereas the control group received the same volume of normal saline. The dosage was set according to preliminary experiments (data not shown). The chronic myocardial injury model included 16 groups, as outlined in Table I). DOX was administered at 1, 2 and 3 mg/kg, named as low-, medium-, and high-dose groups, respectively. All animals received DOX or normal saline once/week via caudal vein injection; the dosages were used as previously described (29,31,32). The clinical symptoms of the animals from both acute and chronic myocardial injury models were observed twice a week. All experiments were approved by the Institutional Animal Care and Use Committee (approval nos. IACUC-2015-P13 and IACUC-2017-K007 for acute and chronic myocardial injury model, respectively) of National Center for Safety Evaluation of Drugs (Beijing, China). Acute and chronic experiment animals were tested once each.

In the acute myocardial injury study, one animal died 48 h after DOX was administered. The time point of 48 h was used to confirm whether the acute myocardial injury model was successfully established; therefore, animals at this time point only underwent histopathological examination. In the chronic myocardial injury model, after 7 weeks, one animal died in groups 15 and 16; therefore, further administration was terminated for all the remaining animals, which were dissected after 8 weeks.

At the designated timepoints, the rats were anesthetized with 4.5% pentobarbital sodium (45 mg/kg) and 3–4 ml blood was collected from the vena cava caudalis, after which the animals were euthanized by exsanguination; death was confirmed by lack of nerve reflex and muscle relaxation. Whole blood was centrifuged at 4°C and 2,000 \times g for 10 min; the serum was collected and frozen at -80°C for later use. The heart was cut longitudinally. One part of the left ventricle was frozen in liquid nitrogen and stored at -80°C for detection of miRNA, whereas the remaining part was fixed with 10% neutral formalin at room temperature for 2 weeks for pathomorphological examination. In the acute myocardial injury model, small RNA sequencing and reverse transcription-quantitative qPCR (RT-qPCR) analysis of heart samples were performed at 24 h after administration.

Serum biochemical detection. CK and LDH activity was quantified using CK (Cat#: 990-64293/996-64393, Wako Pure Chemical Industries, Ltd) and LDH (Cat#: 994-63093/990-63193, Wako Pure Chemical Industries, Ltd) test kits on HITACHI 7180 biochemical analyzer (Hitachi, Ltd.). Serum cTnI, cTnT and FABP3 levels were detected using the MILLIPLEX® MAP Rat Cardiac Injury Magnetic Bead Panel 1 kit (Cat#: RCI 1MAG-87K, EMD Millipore Corporation, Merck KGaA) on a Luminex® 200 platform (Luminex Corporation). cTnI, cTnT and FABP3 concentrations

Table I. Overview of experimental design.

A, Myocardial injury model (acute)						
Group	n	Compound	Dose, mg/kg	Volume, ml/kg	Treatment time, h	Blood/tissue collection time, h
1	5	Saline	0	10	24	24
2	5	DOX	40	10	2	2
3	5	DOX	40	10	4	4
4	5	DOX	40	10	8	8
5	5	DOX	40	10	24	24
6	4	DOX	40	10	48	48 ^a
B, Myocardial injury model (chronic)						
Group	n	Compound	Dose ^b mg/kg	Volume ml/kg	Treatment time (weeks)	Blood/tissue collection (weeks)
1	5	Saline	0	2	2	2
2	5	DOX	1	2	2	2
3	5	DOX	2	2	2	2
4	5	DOX	3	2	2	2
5	5	Saline	0	2	4	4
6	5	DOX	1	2	4	4
7	5	DOX	2	2	4	4
8	5	DOX	3	2	4	4
9	5	Saline	0	2	6	6
10	5	DOX	1	2	6	6
11	5	DOX	2	2	6	6
12	5	DOX	3	2	6	6
13	5	Saline	0	2	7	8
14	5	DOX	1	2	7	8
15	5	DOX	2	2	7	8
16	5	DOX	3	2	7	8

^aNo blood was collected at this time point. ^b1, 2 and 3 mg/kg are low-, medium-, and high-dose groups, respectively. DOX, doxorubicin.

were analyzed using MILLIPLEX software (version 5.1; Merck KGaA).

Sequence analysis of miRNAs. Five animals from the control group and five from the DOX-treated acute myocardial injury model group at 24 h after administration were selected for cardiac miRNA sequencing. A sufficient amount (~50 mg) of myocardial tissue was ground with liquid nitrogen to extract RNA. Following dilution (10-20 times), the concentration, integrity were detected using Agilent RNA 6000 Nano Kit (Cat. no. 5067-1511; Agilent) on a Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.), and purity were evaluated on micro-spectrophotometer (K5500, Chongqing Keao Biotechnology). The sequencing results were validated by RT-qPCR, the extracted RNA was partly used for sequencing and partly used for RT-qPCR validation. A library was constructed using the TruSeq Small RNA Library Prep kit (Cat#: RS-200-0024, Illumina), qPCR was used to accurately

quantify the effective concentration of the libraries (effective library concentration >2 nM) to ensure the quality of the libraries. The files were pooled and sequenced on the Hiseq 2500 system to generate 50 bp long single-end reads. Clean data (18-30 nt) were obtained from high-throughput sequencing through data processing, such as joint, low quality and pollution removal. The sequence length distribution and common sequences between samples were analyzed. The clean data were classified and annotated to obtain information on RNA components and expression levels in the samples. All clean data were annotated according to priority (rRNA>etc> known miRNA > repeat > exon > intron). Unannotated fragments were screened for novel miRNA prediction and differential miRNA expression. Differential miRNA analysis was performed using edgeR software (V3.3, bioconductor.org/packages/3.3/bioc/html/edgeR.html). LogFC >1 or LogFC <-1 && P-value<0.05 were considered to indicate a statistically significant difference.

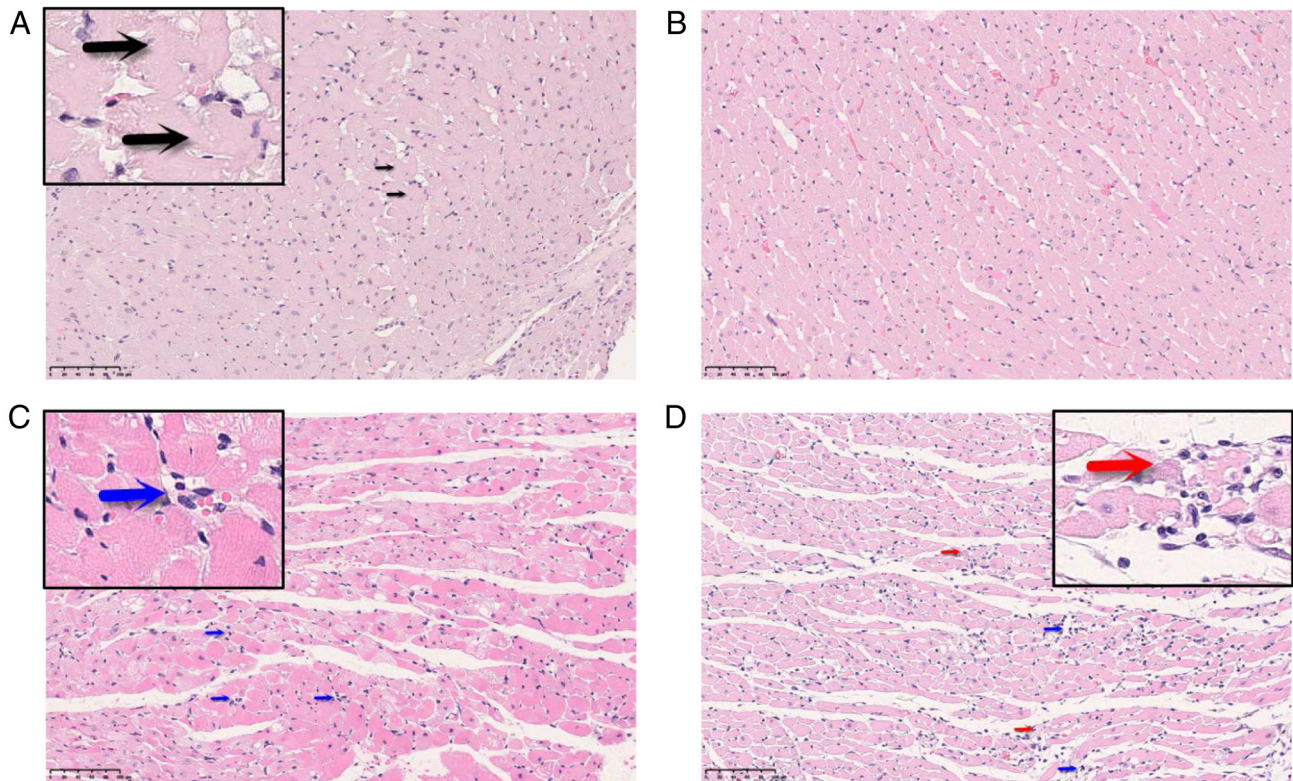


Figure 1. Histopathological changes in the myocardium. (A) Cardiomyocyte degeneration (black arrow) in the DOX-treated (40 mg/kg) group in the acute myocardial injury model following 48 h treatment. (B) No obvious changes in control animal in the chronic myocardial injury model at 6 weeks. (C) Inflammatory cell infiltration (blue arrow) in the ventricle of a DOX-treated (3 mg/kg) chronic myocardial injury model rat at 4 weeks. (D) Extensive cardiomyocyte necrosis (red arrow) and inflammatory cell infiltration (blue arrow) in the ventricle of a DOX-treated (3 mg/kg) chronic myocardial injury model rat at 6 weeks. Scale bar, 100 μ m. DOX, doxorubicin

Quantitative detection of miRNAs by RT-qPCR. miRNAs were extracted and purified from 50-100 mg heart tissue using the EasyPure miRNA kit (TransGen Biotech Co., Ltd.) according to the manufacturer's instructions, and small RNA (≤ 200 nucleotides) was collected. miRNAs were specifically adsorbed on the silica gel membrane of the spin columns provided in the kit; miRNAs were then collected by eluting with 50 μ l RNase-free water. RT was performed using TransScript miRNA First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech Co., Ltd.). qPCR detection was performed using TransStart Top Green qPCR SuperMix kit (TransGen Biotech Co., Ltd.) on the CFX96 Real-Time System Detection Platform (Bio-Rad Laboratories, Inc.). Thermocycling conditions were as follows: Initial denaturation at 95°C, 2 min; stage 2, reaction, repeats: 40, 95°C, 10 sec, 60°C, 15 sec, 72°C, 20 sec. RNU6B (U6) was used as the loading control; sequence specific primers are listed in Table SI. Cq values were calculated using Bio-Rad CFX Manager software (V3.1; Bio-Rad Laboratories, Inc.); the relative expression of miRNAs was calculated using the $2^{-\Delta\Delta Cq}$ method (33).

Histopathological examination. The portion of rat heart fixed with formalin, aforementioned, was embedded in paraffin, sliced into sections (3-5 μ m) and stained with hematoxylin for 5 min and 0.5% eosin for 2 min in the room temperature. Non-blinded examination was performed using a light microscope by two independent pathologists. The degree and extent of lesion were recorded as follows: '-' was defined as lesion not

observed; '+' was defined as minimal severity of lesion; and '++' was defined as mild severity of lesion.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Normality of the data was tested using the Shapiro-Wilk normality test. Nonparametric data with multiple comparisons were analyzed by Kruskal-Wallis; the Mann-Whitney U test was used for comparisons between two groups. Data with normal distribution were analyzed by one-way ANOVA with Dunnett's post hoc test (homogeneous) and Games-Howell (heterogeneous) for multiple comparisons. SPSS 19.0 (version 19.0; IBM Corp) was used to analyze data. $P < 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 8 (GraphPad Software, Inc.) was used to plot data and to perform receiver operating characteristic (ROC) curve and Spearman's correlation analysis. The increase in markers is expressed as effect size, effect size was calculated based on standardized difference (Cohen effect size d) between treatment and control groups using the standard deviation of control groups, which is calculated as follows:

$$\text{Effect Size} = \frac{\text{Mean of the treated group} - \text{Mean of the control group}}{\text{SD of the control group}}$$

Results

Histopathological examination and changes in serum biochemical indicators. In the acute myocardial injury model,

Table II. Histopathology of DOX-treated rats.

A, Myocardial injury model (acute)				
DOX, mg/kg	Interval, h			
	2-4	8	24	48
0	n/a	n/a	- (5/5)	n/a
40	- (5/5)	- (5/5)	- (5/5)	Inflammatory cell infiltration, cardiomyocyte degeneration and necrosis; ++ (3/3) ^a
B, Myocardial injury model (chronic)				
DOX, mg/kg	Interval, weeks			
	2	4	6	8
0	- (5/5)	-(5/5)	- (5/5)	- (5/5)
1	- (5/5)	- (5/5)	Inflammatory cell infiltration; + (1/5)	Cardiomyocyte degeneration; + (2/5) Cardiomyocyte degeneration and necrosis; + (1/5)
2	- (5/5)	- (5/5)	Inflammatory cell infiltration; + (1/5) Cardiomyocyte degeneration; + (4/5)	Inflammatory cell infiltration, cardiomyocyte degeneration and necrosis; ++ (4/4) ^a
3	- (5/5)	Inflammatory cell infiltration; + (1/5) Cardiomyocyte degeneration; + (2/5)	Cardiomyocyte degeneration; ++ (3/5) Cardiomyocyte degeneration and necrosis; ++ (2/5)	Inflammatory cell infiltration, cardiomyocyte degeneration and necrosis; ++ (4/4) ^a

^aOne animal died before euthanasia and no histopathological examination was performed. -, lesion not observed, +, minimal lesion; ++, mild lesion. DOX, doxorubicin. n/a, not applicable.

pathological changes occurred 48 h after administration, including inflammatory cell infiltration, extensive cardiomyocyte degeneration and necrosis (cardiomyocyte degeneration was shown in Fig. 1A; Table II). CK increased between 8 and 24 h after administration (Fig. 2A); neither CK nor LDH was significantly elevated compared to the control group at 24 h.

In the chronic myocardial injury model, the morphological changes were mainly as follows: after 4 weeks of administration, monocyte infiltration and cardiomyocyte necrosis (Fig. 1C; Table II), whereas the pathological changes appearing after 6 weeks were cardiomyocyte degeneration and necrosis (Fig. 1D; Table II). CK and LDH levels in the low-dose group increased significantly after 4 weeks of administration and decreased to levels comparable with those of the control group after 8 weeks (Fig. 2C and D). No significant elevation in CK and LDH was observed in the medium- and high-dose groups. The increase in CK was smaller in the chronic injury model (Maximum effect size=2.15) compared in the acute injury model (effect size=7.04, Table III). Based on the pathological findings, the acute and chronic myocardial injury models were fully established.

Changes in serum cTn and FABP3 levels. In the acute myocardial injury model, cTnI peaked at 8 h after administration,

followed by a decrease to control levels at 24 h (Fig. 3A); cTnT increased at 4 h and continued to rise up to 8 h after administration (Fig. 3B). Neither cTnI nor cTnT was significantly elevated compared to the control group at 24 h. FABP3 increased at 8 h and continued to rise up to 24 h after administration, and significantly elevated at 24 h (Fig. 4A). In the chronic myocardial injury model, cTnI in the low-dose group increased significantly at 8 weeks (effect size=13.14; Fig. 3C and Table III). In the medium- and high-dose groups, cTnI increased significantly at 2 weeks compared with the control (Fig. 3C). However, compared with control, cTnT did not increase significantly in any of the treatment groups (Fig. 3D). A significant increase in FABP3 was only observed in the medium-dose group at 8 weeks compared with the control (Fig. 4B).

Changes in miRNA expression. The sequencing results showed a total of 15 differentially expressed miRNAs were screened (Fig. S1) and five miRNAs were significantly upregulated: miR-122, miR-132, miR-146b, miR-29b and miR-31a (Fig. 5A). As verified by RT-qPCR, miR-122 and miR-146b expression were upregulated and results were consistent with the sequencing results. In the chronic myocardial injury model, 15 differentially expressed miRNAs were

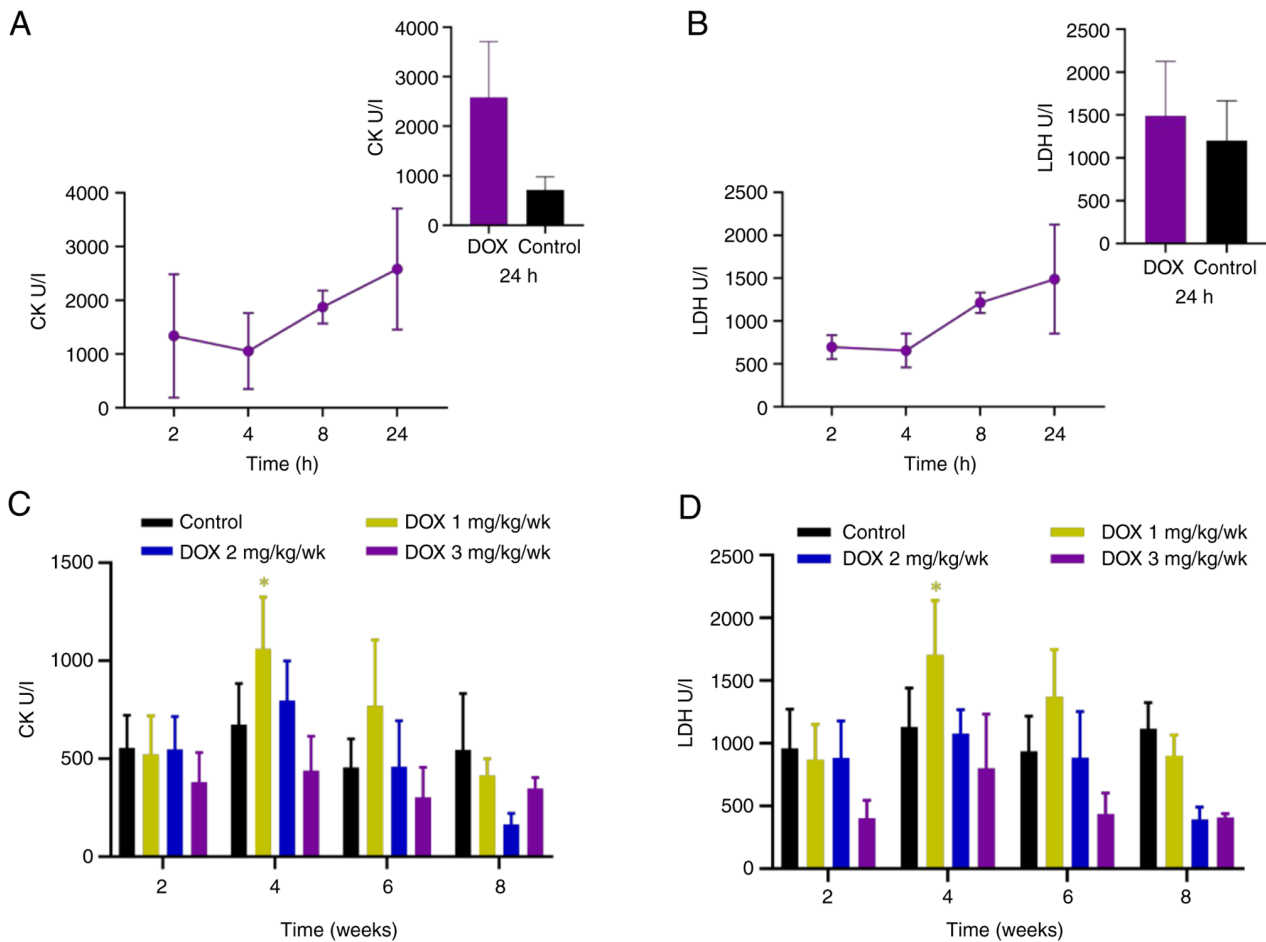


Figure 2. Time course of changes in CK and LDH in rats treated with DOX. Changes in serum (A) CK and (B) LDH levels in the acute myocardial injury model over time; the insets represent comparisons with the control group at 24 h. A significant outlier (6,694U/l, DOX-treated group at 24 h) was excluded for CK in the acute myocardial injury model. Changes in serum (C) CK and (D) LDH levels in the chronic myocardial injury model rats. * $P < 0.05$ vs. control. CK, creatine kinase; DOX, doxorubicin; LDH, lactate dehydrogenase.

analyzed by RT-qPCR in the control and high-dose groups (3 mg/kg) at 6 weeks. A total of six miRNAs were significantly upregulated: miR-122, miR-146b, miR-150, miR-21, miR-34a and miR-144, whereas miR-31a was significantly downregulated (Fig. 5B). miR-146b, which is associated with cardiotoxicity (34), was chosen as a candidate marker; the other candidate marker was miR-208a, which is reported to be associated with cardiotoxicity (29).

In the acute myocardial injury model, relative expressions of miR-146b and miR-208a increased between 4 and 24 h (Fig. 6A and B), however, expression of miR-146b was lower than that in the control group at 24 h, and the expression of miR-208a was slightly higher than that in the control group. In the chronic myocardial injury models, the miR-146b and miR-208a levels in the low-dose group were lower compared with those in the control group at 2 weeks; the relative expression of miR-146b in the low-dose group increased at 6 weeks, whereas relative expression of miR-208a increased at 4 weeks. In the medium- and high-dose groups, miR-146b and miR-208a increased at 2 weeks. The relative expression of miR-146b in the medium-dose group increased significantly at 8 weeks compared with the control group (Fig. 6C); while expression of miR-208a in the medium-dose group was significantly elevated at 2 weeks (Fig. 6D).

ROC and correlation analysis. In ROC analyses, pathological changes were considered to indicate toxicity; absence of pathological changes was considered non-toxic. ROC and Spearman's correlation analyses were performed only for chronic myocardial injury at weeks 6 and 8. Biomarkers were compared by ROC analysis using area under the curve (AUC) to evaluate the diagnostic performance (Fig. 7). CK (AUC=0.71), LDH (AUC=0.76) and FABP 3 (AUC=0.70) exhibited high predictive value at 6 weeks, of which, CK and LDH were negatively correlated with myocardial pathological injury ($\rho = -0.43$ and $\rho = -0.49$; Fig. S2), and FABP 3 was positively correlated with myocardial pathological injury ($\rho = 0.24$). At 8 weeks, cTnI (AUC=0.83; $\rho = 0.55$) and miR-146b (AUC=0.71; $\rho = 0.50$) showed a relatively high predictive value and strong correlation with myocardial pathological injury. The predictive value of FABP 3 at 8 weeks was also relatively high (AUC=0.79) and exhibited a moderate correlation with pathological injury ($\rho = 0.38$), other markers negatively correlated with myocardial injury.

Discussion

One of the primary side effects of DOX is cardiotoxicity (32). Therefore, DOX is used to establish experimental models of

Table III. Effect size^a of biomarkers.

A, Myocardial injury model (acute)								
Interval, h	DOX, mg/kg	CK	LDH	cTnI	cTnT	FABP3	miR-146b	miR-208a
24	40	7.04	0.62	-0.25	0.34	6.09 ^b	-0.42	0.24
B, Myocardial injury model (chronic)								
Interval, weeks	DOX, mg/kg	CK	LDH	cTnI	cTnT	FABP3	miR-146b	miR-208a
2	1	0.79	0.62	0.00	0.61	0.67	-0.81	-0.65
	2	-0.34	-0.61	9.56 ^c	-0.15	-0.44	1.38	3.27 ^b
	3	-1.10	-1.86	11.04 ^c	-0.23	-0.36	3.33	3.54
4	1	1.33 ^b	1.37 ^c	-0.13	-0.41	-0.52	-0.20	0.34
	2	2.05	0.32	3.56 ^c	0.30	3.08	0.76	0.53
	3	-0.74	-0.74	-6.06	-0.22	1.09	-0.41	0.27
6	1	2.15	1.56 ^c	0.95	0.36	0.18	0.18	-0.89
	2	0.02	-0.18	1.46	0.01	1.06	-0.45	-0.87
	3	-1.05	-1.80	3.86	-0.12	1.99	0.21	0.17
8	1	-0.46	-1.04	13.14 ^c	0.27	0.02	-1.94	0.97
	2	-1.32	-3.49	30.24	-0.60	5.28 ^b	4.64 ^b	-1.40
	3	-0.69	-3.41	6.92	-1.17	0.37	1.60	-1.22

^aEffect size was calculated based on standardized difference (Cohen effect size d) between treatment and control groups using the standard deviation of control group. ^bP<0.05, ^cP<0.01 vs. control. CK, creatine kinase; cTn, cardiac troponin; DOX, doxorubicin; FABP3, fatty acid-binding protein 3; LDH, lactate dehydrogenase; miR, microRNA.

cardiotoxicity (32,35). DOX causes acute, as well as chronic, myocardial injury. The present study identified five biomarkers to evaluate these injuries, including CK, LDH, cTn, FABP3 and miRNA. To the best of our knowledge, most reports (8,12,29) have only evaluated changes in one or two of these markers in a single injury model. Markers vary in sensitivity and specificity to different toxicity models and should therefore be evaluated in detail. The present study evaluated changes in myocardial injury markers in both acute and chronic myocardial injury models.

Pathological changes, such as inflammatory cell infiltration, cardiomyocyte degeneration and necrosis, are considered to indicate successful induction of a rat myocardial injury model. The present study aimed to determine changes in miRNA, as well as pathological injury at different time points. Therefore, expression levels of miRNA markers were investigated before the occurrence of pathological injury, therefore, sequencing analysis was performed 24 h after administration in the acute myocardial injury model, the aim was to find potential markers for the early prediction of the myocardial injury. P-value was used to reflect the statistical significance of toxicity, whereas the effect size was used to reflect the degree of toxicity (36). The LDH level did not elevated in the high-dose group, but was lower than the control group at 2 weeks; cTnT, miR-146b and miR-208a levels did not elevated in the low-dose group, but were lower compared with those in the control group at 2 weeks, which may be influenced by inter-individual differences in the animals.

When cardiomyocytes are injured, intracellular enzymes are released into the blood, therefore, these enzymes serve as markers for myocardial injury (37,38). Numerous studies (39-41) have shown that DOX treatment leads to increased serum CK and LDH levels. In the present acute myocardial injury model, CK began to rise at 8 h following DOX administration, which was earlier than cardiac histopathological changes, which occurred 48 h after administration. In the chronic injury model, CK and LDH in the low-dose group increased significantly after 4 weeks, but the increase in CK and LDH levels was not observed in the high-dose group. Fredericks *et al* (42) showed that the half-life of CK in rats is 0.6 h, suggesting that the increase in CK level in the high-dose group may occur 2 weeks earlier than that in the chronic injury model. The increase in CK in the acute injury model was larger than that in the chronic myocardial injury model. Therefore, CK was relatively sensitive to acute myocardial injury. In addition, owing to the short half-life of CK and its rapid clearance *in vivo*, the blood must be taken prior to clearance.

Although the diagnostic performance of CK and LDH were good, they were negatively correlated with myocardial injury and showed poor specificity. Other tissue damage, such as skeletal muscle, can also lead to an increase in CK and LDH levels (43,44).

cTn is a regulatory globular protein found in thin myofilaments that is involved in myocardial contraction (16). In the acute injury model, cTnI peaked at 8 h then decreased. However, the time taken to peak by cardiotoxic compounds

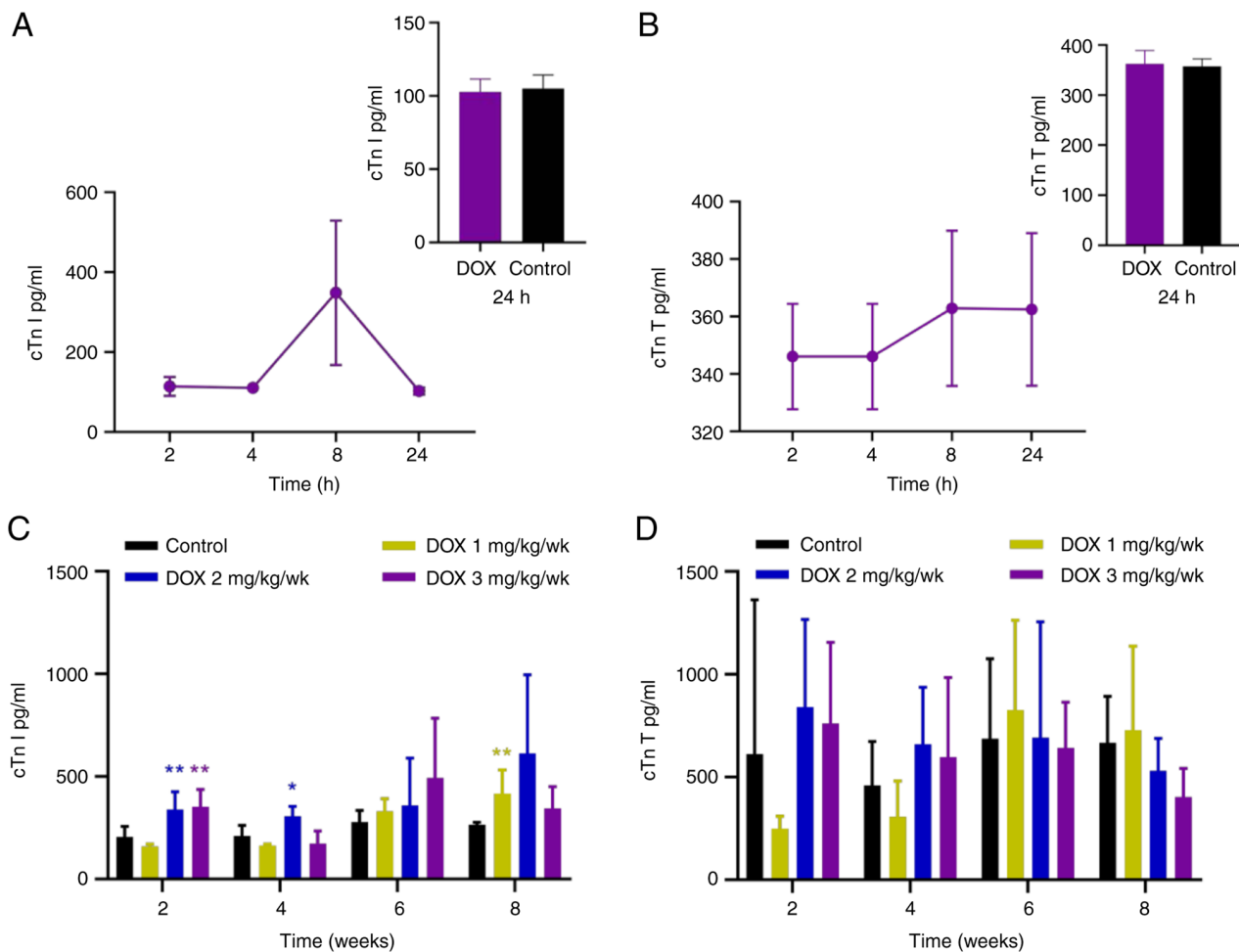


Figure 3. Serum cTnI and cTnT levels following treatment with DOX. Changes in serum (A) cTnI and (B) cTnT levels in the acute myocardial injury model. A significant outlier (1,308 pg/ml, DOX-treated group at 8 h) was excluded for cTnI in the acute myocardial injury model. Changes in serum (C) cTnI and (D) cTnT levels in the chronic myocardial injury model. * $P < 0.05$, ** $P < 0.01$ vs. control. cTn, cardiac troponin; DOX, doxorubicin.

varies. A previous study showed that cTnI rises 1 h after administration of isoproterenol (1.5 mg/kg), peaks at 2 h and returns to baseline level at 72 h (45). By comparison, cTnI increases significantly at 72 h after DOX (20 mg/kg) administration. Therefore, cTnI may be an early indicator of DOX-induced acute myocardial injury. However, the elevation of cTnI is drug-specific (46).

In the chronic myocardial injury model, the increase in cTnI in the low dose-group was noted at 6 weeks, whereas the increase in the middle- and high-group occurred at 2 weeks after administration, indicating that a higher dose induced an earlier increase in cTnI. Reagan *et al* (32) showed that the degree of cTnI elevation and the incidence of cTnI elevation increased with increasing dose, as well as with longer dosing cycles, and some animals showed a decrease in cTnI after discontinuing DOX administration. Serum cTnI levels are maintained for 5-7 days after elevation before being cleared (47). In the present study, at 2 weeks, circulating cTnI increased significantly but no histopathological changes were noted, suggesting that cardiomyocytes were damaged which led to release of intracellular free or partially-conjugated cTnI into the blood. At 4 weeks, cTnI levels in some animals (3/5 in the medium- and 5/5 in the high-dose group) decreased, potentially owing to faster clearance rate. At 6 weeks, pathological

changes in cardiomyocytes worsened and the lesions were enlarged. Massive cardiomyocyte necrosis (mild severity of lesion) followed release of numerous conjugated cTnI into the blood, along with a corresponding increase in circulating cTnI. The largest increase in cTnI was observed in the medium-dose group and was greater than that of cTnT. Reagan *et al* (32) showed that the rate of cTnI production in rat serum was greater than that of cTnT. At 8 weeks, the most serious pathological injury of cardiomyocytes was noted and cTnI showed the best predictive potential for myocardial injury. Furthermore, it was positively correlated with myocardial pathological injury, indicating a higher sensitivity to structural damage of cardiomyocytes. Because free cTnI in the cytoplasm is low (48), a significant increase indicates structural damage. cTnI increased earlier than cTnT and FABP 3 in the chronic myocardial injury model, the degree of change was greater and elevated for a longer duration, suggesting that good sensitivity and a wide detection window for a marker.

In the present study, cTnT increased 8 h following treatment with a single high dose of DOX. Wu and Feng (49) hypothesized that the first peak in the increase in cTn in peripheral blood is due to cTnT in acute coronary syndrome, because 6-8% of free cTnT exists in the cytoplasm, whereas cTnI seldom exists in the free form (2.8-4.1%) and thus increases

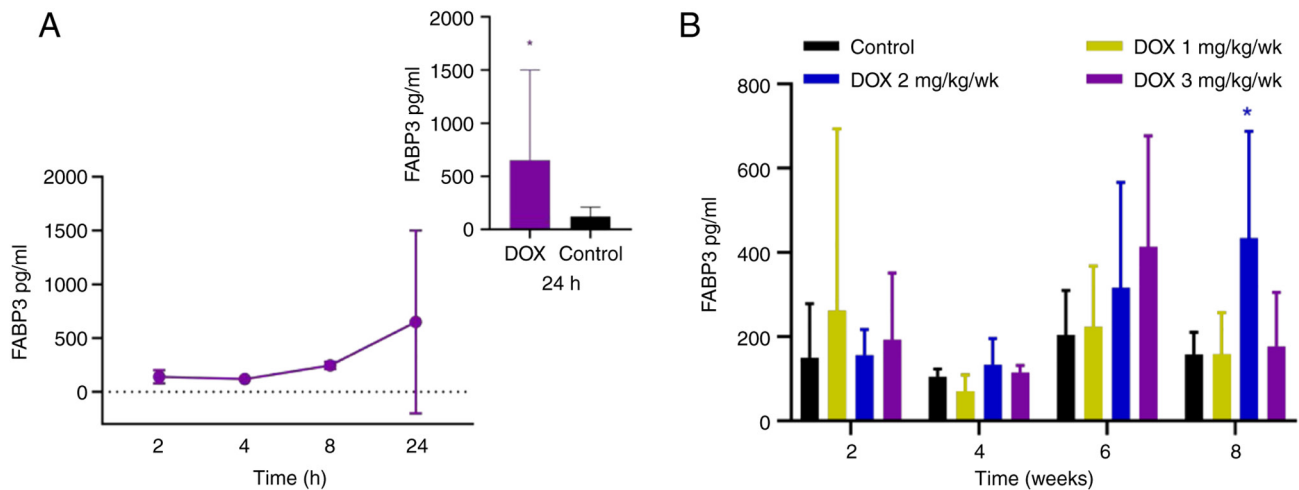


Figure 4. Serum FABP3 levels following treatment with DOX. Changes in serum FABP3 level in (A) acute and (B) chronic myocardial injury model rats. *P<0.05 vs. control. DOX, doxorubicin; FABP3, fatty acid-binding protein 3.

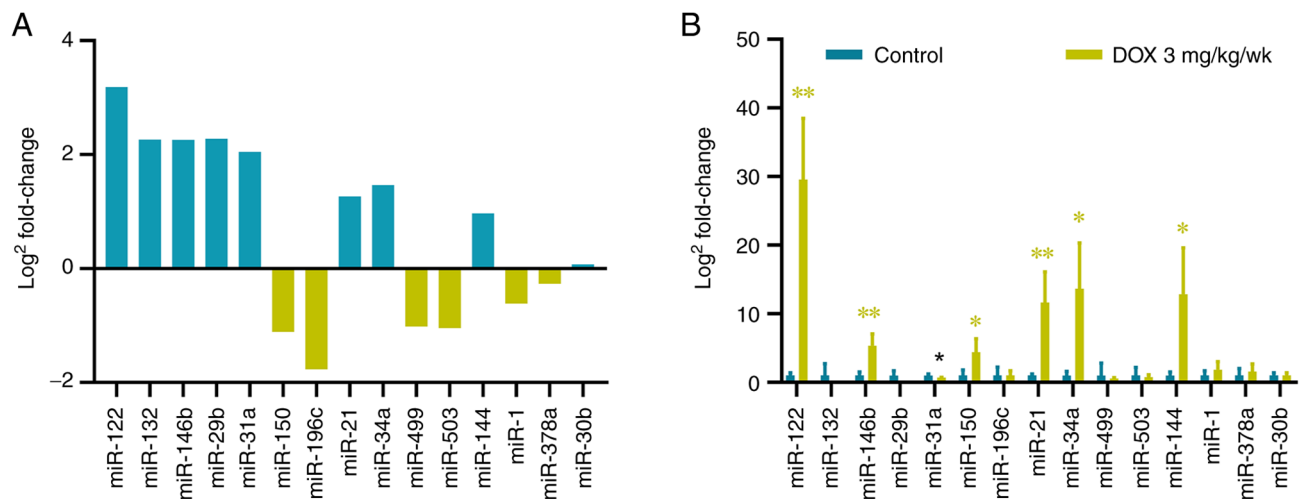


Figure 5. Differential expression of miRs between control and DOX groups. Differentially expressed miRs of sequencing and qPCR assay results in (A) acute and (B) 6-week chronic myocardial injury model. Mann-Whitney U test was used for comparison. *P<0.05, **P<0.01 vs. control. miR-31a was significantly downregulated. DOX, doxorubicin; miR, microRNA.

later than cTnT (48). In the present study, the increase in cTnT was not initially observed because compared with conjugated cTnT, free cTnT accounted for a small proportion (48). In addition, owing to the insufficient sensitivity of the assay, a relatively small increase could not be identified. In the chronic injury model, no significant increase of cTnT was observed. In the study by Herman *et al* (50), repeated administration of DOX (1 mg/kg) with cumulative doses of 2 and 4 mg/kg did not increase cTnT. However, when the cumulative dose reached 7 mg/kg, cTnT increased significantly. In the present study, no significant increase in cTnT was observed, which may have resulted from an insufficient administration period.

FABP3, also known as heart-type FABP, is released rapidly (within 1 h) following myocardial injury (51). In the acute myocardial injury model, FABP3 was increased at 8-24 h after administration, and significantly elevated at 24 h. In the chronic myocardial injury model, FABP3 showed a dose-dependent increase at 6 weeks of DOX administration. In the middle-dose group, FABP3 showed a significant increase

at 8 weeks; there were no significant time-dependent changes and the dose-dependent change in FABP3 was not obvious. These results indicated that FABP3 was more sensitive to acute myocardial injury.

In the chronic injury model, the relative expression of miR-146b in the high-dose group increased at 2 weeks, then declined, the results were generally consistent with literature (52). In a previous study of male mice treated with DOX weekly (5 mg/kg), miR-146a expression in myocardial tissue and plasma was upregulated, reaching a peak after 3 days and lasting for 1 week before being cleared (52). The relative expression of miR-146b showed a dose-dependent increase at 2 weeks of DOX administration, this result was consistent with Horie's report (34). Horie *et al* showed that miR-146a expression increases in a dose-dependent manner following DOX treatment in neonatal rat cardiomyocytes, reaching a peak at 16 h after administration (34,52). The pyroptotic marker IL-1 β also increases following DOX exposure and is involved in NLRP3-mediated pyroptosis in H9c2 cells (53).

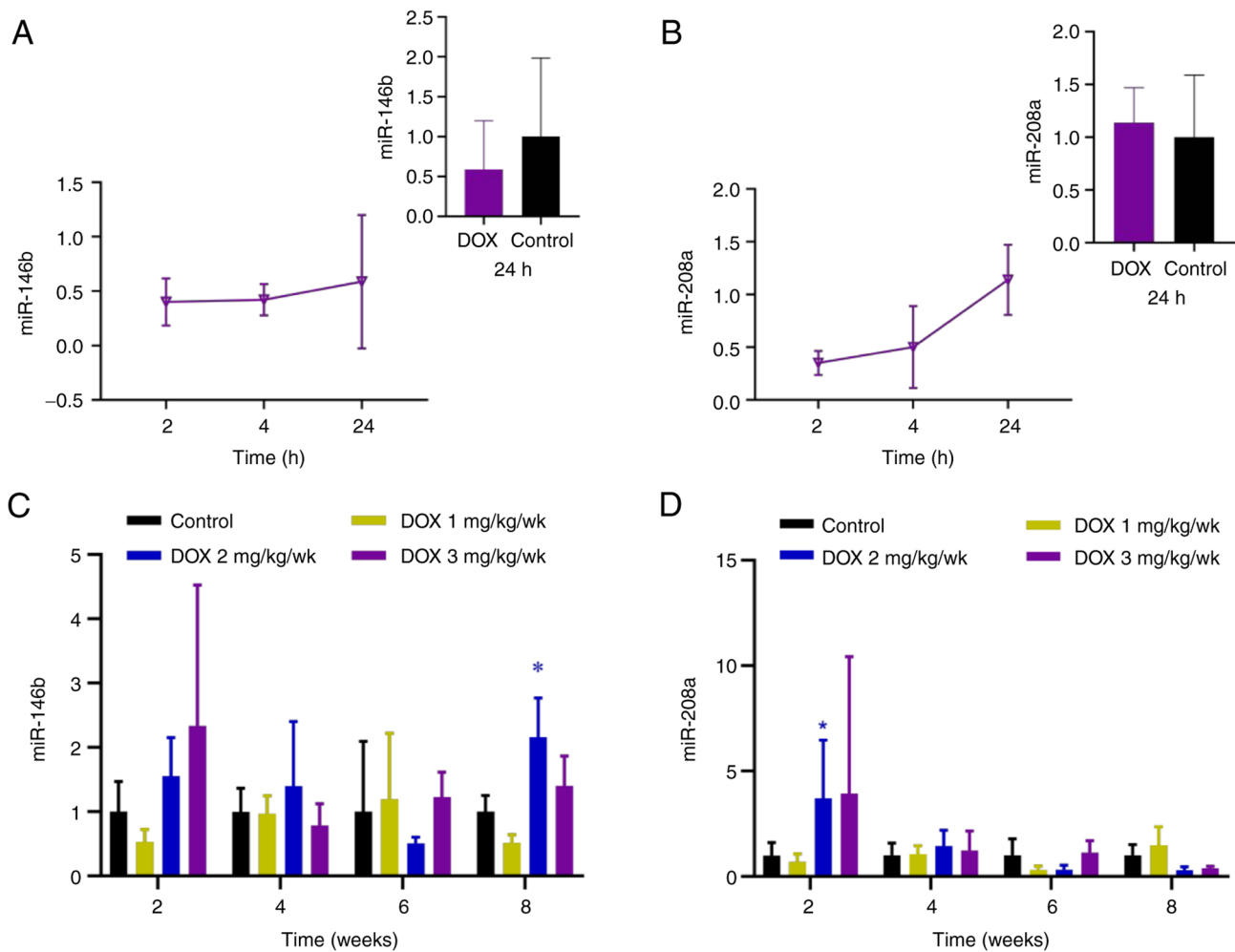


Figure 6. Relative expression levels of miR-146b and miR-208a in myocardial tissue following DOX administration. Change in (A) miR-146b and (B) miR-208a expression in the acute myocardial injury model. Changes in (C) miR-146b and (D) miR-208a expression in the chronic myocardial injury model. Data were not available for rats at 8 h in the acute cardiac injury study owing to RNA degradation. * $P < 0.05$ vs. control. DOX, doxorubicin; miR, microRNA.

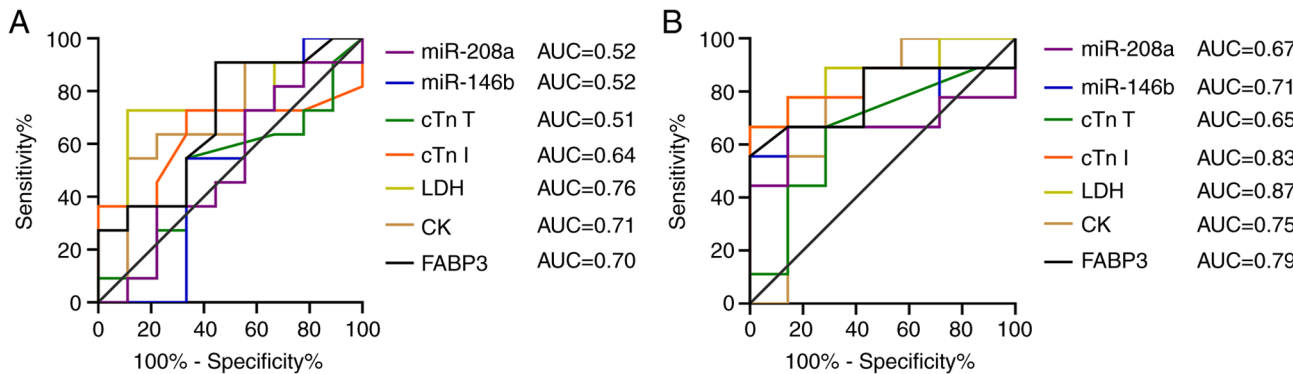


Figure 7. ROC curves for CK, LDH, cTn I, cTnT, FABP3, miR-146b and miR-208a. ROC curves were plotted for chronic myocardial injury at (A) 6 and (B) 8 weeks. AUC, area under the curve; CK, creatine kinase; cTn, cardiac troponin; FABP3, fatty acid-binding protein 3; LDH, lactate dehydrogenase; miR, microRNA; ROC, receiver operating characteristic.

IL-1 receptors are activated by IL-1 β , which activate MAPKs, such as MEK-1/2 and JNK-1/2, which mediate transcription of miR-146b (54,55). Although the mechanism is not clear, IL-1 β may induce miR-146b expression (54,55). miR-146a mediates maintenance of normal function of mature cardiomyocytes by inhibiting the neuregulin/ErbB pathway; it

also inhibits DOX-induced cardiotoxicity by inhibiting the TATA-box binding protein-associated factor 9b/p53 signaling pathway (34,52). In addition, knocking out the miR-146a gene in mice aggravates DOX-induced myocardial injury (52). In the present study, after 8 weeks of DOX administration, miR-146b showed a relatively high predictive value and was

correlated with myocardial pathological injury. Therefore, miR-146b may serve as a marker for DOX-induced chronic cardiotoxicity. However, the significance of the increase in miR-146b needs further study. In the present study, both sequencing results and RT-qPCR validation results showed miR-146b as up-regulated (compared with the control), while the expression of miR-146b in the acute injury model was lower than that in the control group, this may be due to the fact that the RNA used for the PCR assay was not the same batch as the sequencing sample, resulting in a slightly lower expression level in the DOX-treated group (Mean=1.00) than in the control group (Mean=1.07).

miR-208a is specifically expressed in the heart and is a regulator of cardiac hypertrophy and the cardiac conduction system (45,56). In the present study, acute myocardial injury mode, miR-208a increased slightly at 24 h after administration compared with the control group. The changes of miR-208a for acute myocardial injury is also compound-specific. Isoproterenol (1.5 mg/kg intraperitoneal) significantly increases miR-208a within 1 h, with a peak at 4 h (45). miR-208a reaches peak value at 24 h after administration of allylamine (100 mg/kg) (45). In the chronic myocardial injury mode, miR-208a in the medium-dose group increased significantly at 2 weeks. The changes of miR-208a for chronic myocardial injury may be compound-specific. In the report of nishimura's, following repeated administration of isoprenaline in rats (0.5 mg/kg), the relative expression of miR-208a in plasma increases significantly after 2 days and decreases after 4 days (29). Sadek *et al* (57) showed that fibrogenic factors, such as α smooth muscle actin, TGF- β 1 and p16^{INK4A}, are upregulated following DOX-induced cardiac injury. TGF- β 1 binds to type II TGF- β receptor, subsequently activating type I TGF- β receptor. The activated type I TGF- β receptor phosphorylates SMAD proteins, which transduce the signal to the nucleus, thereby increasing activity of the miR-208a promoter (58). The upregulation of miR-208a is induced by DOX. One target of miR-208a is GATA4; increased miR-208a expression downregulates GATA4, resulting in cardiomyocyte apoptosis and heart dysfunction (34). Furthermore, miR-208a silencing attenuates myocardial apoptosis (30). These results suggested that miR-208a could serve as a potential biomarker for DOX-induced cardiotoxicity.

One limitation of the present study is the small sample size, which limits the generalizability of findings and ROC analysis. Here, only DOX-induced cardiotoxicity was evaluated. Whether the seven biomarkers evaluated were DOX-specific or not and whether they have predictive value for other compounds needs further investigation.

In conclusion, the present study showed that CK, cTnI and FABP3 were relatively sensitive markers for DOX-induced acute myocardial injury, of which CK and FABP3 were elevated to a greater extent than in the chronic injury model. cTnI was relatively sensitive to DOX-induced chronic myocardial injury, cTnI and miR-146b showed relatively high predictive values for late-stage myocardial injury (following occurrence of myocardial pathological changes). Therefore, CK, cTnI and FABP3 may serve as toxicity endpoints for compounds with expected acute myocardial injury, whereas cTnI and miR-146b may serve as toxicity endpoints for compounds with expected chronic myocardial injury.

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Availability of data and materials

The raw sequencing data in this study have been deposited in NBCI's Sequence Read Archive (accession no. PRJNA839543); other data generated or analyzed during this study are included in this manuscript published article.

Authors' contributions

DP, SW and BL contributed to the conception of the study. DP and SW performed the experiments. DP analyzed data and wrote the manuscript. DP and SW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of National Center for Safety Evaluation of Drugs (Beijing, China; approval nos. IACUC-2015-P13 and IACUC-2017-K007).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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