International Immunopharmacology 74 (2019) 105733

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The cardioprotective effects of icariin on the isoprenaline-induced takotsubo-like rat model: Involvement of reactive oxygen species and the TLR4/NF-κB signaling pathway

Chunlei Qi[a], Yangzhen Shao[b,c], Xuesong Liu[a], Daxin Wang[d,e,*], Xueping Li[f]

[a] Department of Cardiology, the Second Xiangya Hospital of Central South University, China
[b] Department of Molecular and Clinical Medicine, Institute of Medicine, Gothenburg University, Sweden
[c] Department of Cardiology, Sahlgrenska University Hospital, Sweden
[d] Clinical Medical College, Yangzhou University, China
[e] Department of Medical Research Centre, Northern Jiangsu People's Hospital, China
[f] Nanjing Hospital Nanjing Medical University, China

ARTICLE INFO

Keywords:
Takotsubo
Icariin
Reactive oxygen species
TLR4/NF-κB signaling pathway

ABSTRACT

Introduction: Takotsubo syndrome (TS) is an acute cardiac syndrome that mimics acute coronary syndrome (ACS) but lacks coronary obstruction and is associated with sudden physical or psychiatric episodes. Several hypotheses have been proposed for the TS mechanism, but the precise cause of this syndrome remains poorly known. Recent studies noted TS patients with acute endogenous catecholamine discharge, which could trigger an oxidative stress response and inflammatory action.

Methods: A single dose of the selective β-adrenergic agonist isoprenaline (ISO) was used to induce a takotsubo-like (TS-like) model. Different icariin or metoprolol doses were supplied as cardioprotective agents by intragastric administration (IG), and lipopolysaccharides (LPS) were assessed to investigate the possible mechanism of action of icariin. Transthoracic echocardiography was used to study cardiac function and morphology. The amounts of intracellular lipids and myocardial fibrosis, which represent the degree of cardiac impairment, were assessed by histological analysis. Real-time polymerase chain reaction (RT-PCR) was performed to analyze a variety of anti-oxidant elements and inflammatory factors, and Western blotting was conducted to analyze the expression of signaling pathway proteins involved in the development of TS.

Results: The TS-like incidence in rats was lowest with icariin precondition at 2-h post-ISO administration, and both the left ventricular ejection fraction (LVEF) and ejection volume per minute were higher than those of the other groups. However, LPS administration increased the incidence of TS and aggravated cardiac impairment. Moreover, ISO significantly increased the levels of both reactive oxygen species (ROS) and TLR4/NF-κB signaling pathway proteins compared to those of the Sha-group, whereas icariin remarkably decreased the ROS levels and increased anti-oxidant element expression while reducing pro-inflammatory factor secretion and suppressing TLR4/NF-κB signaling pathway protein expression. However, the cardioprotective effect of icariin was significantly weakened by combining treatment with LPS.

Conclusion: Icariin prevented ISO-induced TS-like cardiac dysfunction in rats. The effects were induced mainly through maintenance of the dynamic balance of the ROS system, promotion of anti-oxidant element activity, and suppression of TLR4/NF-κB signaling pathway protein expression. Furthermore, the ability of icariin to increase anti-inflammatory and reduce pro-inflammatory factor secretion may be involved in the protective process.

1. Introduction

Takotsubo syndrome (TS), which is also referred to as transient apical ballooning or stress-induced cardiomyopathy, evolves from the psychological or physiological stress response [1–3], such as emotional disorders, traumatic fracture, pulmonary diseases, malignancies. The major characters of TS is associated with convex ST-segment elevation and a moderate increase in the creatine kinase and troponin levels [1,4], which are similar to acute coronary syndrome. To date, the only definitive progress that has been made in elucidating the pathogenesis

* Corresponding author at: Clinical Medical College, Yangzhou University, China.
E-mail addresses: Shao.Yangzhen@wlab.gu.se (Y. Shao), daxinw2016@163.com (D. Wang).

https://doi.org/10.1016/j.intimp.2019.105733
Received 5 February 2019; Received in revised form 26 June 2019; Accepted 29 June 2019
Available online 06 July 2019

of TS is related to the pivotal role of catecholamine release [5]. A number of hypotheses have been proposed, including estrogen deficiency, catecholamine direct myocardial toxicity and coronary microcirculation dysfunction, however, none of which was validated to play a core role in the development. In recent years, the relationship between immune system and TS has aroused huge attention from cardiologists, since reactive oxygen species (ROS) accumulation [6] and downstream inflammatory cells and inflammatory factors [7] are significantly increased in both clinical patients and rodent models. Besides, the major relevant factors of TS, such as mental stress and catecholamine storm, are closely associated with activation of immune system.

ROS are common metabolites that originate mainly from mitochondrial and endoplasmic reticulum metabolism and are known to act as second messengers in cellular activity [8–11]. Clinical and experimental studies have provided substantial evidence that excessive ROS production is tightly connected with cardiomyocyte hypertrophy and dysfunction [12,13]. Growing evidence suggests that excessive ROS production is also associated with pro-inflammatory signaling pathways and inflammatory factor activation [14,15]. Toll-like receptor 4 (TLR4), which is a key proximal signaling receptor that is responsible for initiating the innate immune response, mediates the inflammatory immune response, which is characterized by leucocyte activation and myocardial infiltration. This response leads to cytokine secretion [16–18], which has also been found to be associated with TS development in a rodent model [19].

Flavonoid glucoside, which includes many important substrates extracted from natural plants, has been reported to show strong antioxidant stress potential and inhibit cardiomyocyte functional disturbance [20–22]. Icariin ($C_{33}H_{40}O_{15}$, molecular weight = 676.67), which is the major active ingredient of the Epimedium family, has been proven to have various pharmacological activities, such as anti-atherosclerosis [23], anti-oxidant [24,25], and anti-inflammatory [26,27] effects, both in vitro and in vivo. Thus, we have designed this experiment to test the hypothesis that icariin attenuates isoprenaline-induced TS-like cardiac dysfunction.

2. Methods

2.1. Animals and protocol

All procedures involving rats were approved by the Animal Care and Use Committee of Nanjing Medical University (permit number IACUC-1712006). All procedures were performed in accordance with the standards published by the National Research Council (NIH OACU. Number 86-23) and the National Institutes of Health policy on human care and the use of laboratory animals.

Male Sprague Dawley (SD) rats weighing 300–350 g were purchased from the Vital Animal Laboratories of Nanjing Medical University (Nanjing, China). All rodents were housed in a suite of conventional clean animal (CCV) rooms under identical conditions (12/12 hour light/dark cycle, 24–26 °C, and 40–70% humidity) and received food and water ad libitum. Two protocols were included in the experiment; In protocol 1, 48 rats were randomly divided into five different groups as follows; Sha-group (6 rats, drinking water IG), Con-group (12 rats, drinking water IG), Met-group (12 rats, metoprolol (Asilkan, UK) 10 mg/kg/day IG), and Ica-group (12 rats, icariin (Tianjiang Pharmaceutical Co., Ltd., Soochow, China) 1 g/kg/day IG). To determine whether the protective effect of icariin was mediated through suppression of the TLR4 signaling pathway, one additional group was included, LPS-group (6 rats, icariin 1 g/kg IG + LPS 5 mg/kg (Sigma, USA) IP). The pretreatment lasted one week, and all rats except the Sha-group received a single intraperitoneal injection of ISO (50 mg/kg, in saline, Sigma, USA) on the last day. In protocol 2, 36 rats were randomly divided into three different groups as follows: L-group (icariin 0.5 g/kg IG), M-group (icariin 1 g/kg IG) and H-group (icariin 2 g/kg IG). Icariin pretreatment was performed as described above for one week, and the rats received a single intraperitoneal injection of ISO (50 mg/kg, in saline) on the last day.

2.2. Cardiac function assessment (echocardiography and blood pressure monitoring)

High-resolution transthoracic echocardiography (Visual Sonics, Vevo 3100 imaging station, Canada) was used to evaluate the left ventricular function and morphology of the rats. First, the rats were anesthetized with isoflurane, and the fur in the precardiac-area was removed with an electric clipper and hair-removing gel. Then, a 35 MHz linear transducer and ultrasound probe was applied to obtain an optimal parasternal long axis cine loop, which provided an image that with clearly visible mitral and aortic valves and a maximum distance between the aortic valve and the cardiac apex. All cine loops consisted of N1000 frames/s and were acquired using the ECG-gated kilohertz visualization technique. All rats in the experiment received a blood pressure test, and the heart rate was recorded with a noninvasive blood pressure system tail cuff (Coda 4.1, USA) at 1, 2, 3, 4, 5 and 6-h post-ISO injection.

2.3. Biochemical index activity assays

Blood samples obtained from the abdominal aorta of rats in each group were centrifuged at 3500*g for 10 min at 4 °C and stored at −80 °C as quickly as possible. The plasma levels of the myocardial enzyme spectrum were measured with an automatic biochemical analyzer (Olympus AU640, Japan). All measurements were performed according to the manufacturer's instructions.

2.4. Histological analysis

All rats in the experiment were sacrificed within the first 24 h after ISO injection, and cardiac tissue from the akinesis segment of the left ventricle or apical segment was removed immediately and fixed in a 10% formalin solution. The heart tissue was processed for sectioning and staining using standard histological methods, including Masson's trichrome and oil red O staining, and images were obtained by microscopy (ScanScope CS, Aperio, Olympus). The lipid contents and extent of fibrosis (blue-green fibrosis) were quantified using the ImageJ 1.48u software.

2.5. Measurement of TBARS levels and SOD activity and ROS generation

The level of serum level of thiobarbituric acid-reactive substances (TBARS) were measured [28] using Assay Kit (AmyJet Scientific Inc. Ltd., Wuhan, China). Superoxide dismutase (SOD) activity was measured using the Superoxide Dismutase Activity Assay Kit (Abcam, ab65354) according to the manufacturer's instruction. Frozen sections from the different groups were incubated with 10 mM dihydroethidium (DHE) (Invitrogen) for 30 min at 37 °C in the dark. After incubation, the fluorescence of the tissues was captured by a fluorescence microscope. The ROS signals were quantified by the Image-Pro Plus6.0 (Media Cybernetics, Rockville, MD, USA).

2.6. RT-PCR

Selected gene transcripts were subjected to real-time quantitative

polymerase chain reaction (RT-PCR) analysis. First, the target gene RT-PCR primers was selected using sequences from the PrimerBank database: IL-1β, 5′-GGCAACTGTCCCTGAACTC AAC-3′ (forward) and 5′-AAGCTCCACGGGCAAGACATA-3′(reverse); IL-6, 5′-A AGAGACTTC CAGCCAGTTGCC-3′ (forward) and 5′-TGTGGGTGGTATCCTCT GTGAAG-3′(reverse); TNF-α, 5′-CCAGGTTCTCTTCAAGGGACAA-3′ (forward) and 5′-GGTATGAAATGGCAAATCGGCT-3′(reverse); sod1, 5′-GCGTCATTCACT TCGAGCAG-3′ (forward) and 5′-GGACCGCCATGT TTCTTAGAGT-3′(reverse); cat, 5′-TCTCCATCAGGTTACTTTCTTG TTC-3′ (forward) and 5′-ATGCCCTGGT CAGTCTTGTAATG-3′ (reverse); GAPDH, 5′-CTGGAGAAACCTGCCAAGTATG-3′ (forward) and 5′-GGTG GAAGAATGGGAGTTGCT-3′ (reverse). cDNA was synthesized for 50 min at 50 °C in a 20 μl reaction containing 1× First-Strand Buffer, 300 ng of total RNA, 200 ng of random hexamer oligonucleotides, 10 mM DTT, 0.5 mM dNTPs, 40 units of RNase inhibitor and 200 units of Superscript II reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany). Then, the RT-PCR in a 25 μl reaction were performed using a 96-well format (1.0 μl of cDNA, 200 nM of each primer, and 1 × SYBR Green Super Mix) by a Icycler real-time PCR system (Bio-Rad, Munich, Germany). Five samples were measured in each experimental group in triplicate from a minimum of two independent experiments. The relative amount of target mRNA normalized to HPRT1 was calculated according to the method described by Pfaffl.

2.7. Western blotting analysis

Western blotting was performed according to the manufacturer's instruction. Proteins were extracted from each experiment (30 μg per lane), homogenized in lysis buffer and separated by 10% SDS-PAGE. The protein concentration was measured using a BCA kit (Abcam, USA). The separated proteins were transferred to nitrocellulose membranes. After incubation in blocking solution (5% nonfat dry milk), the

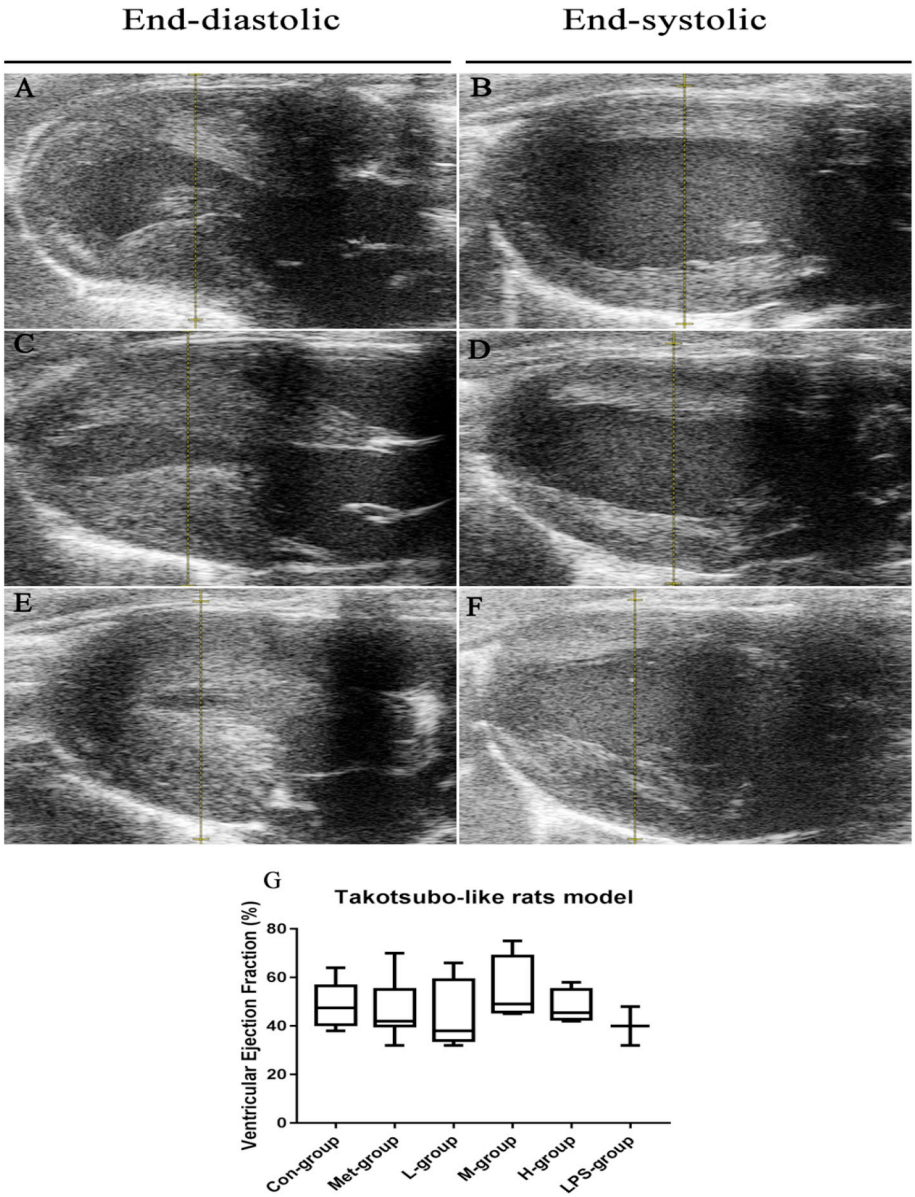

Fig. 1. End-diastolic (A) and end-systolic (B) echocardiographic images of one rat that developed typical TS-like dysfunction 2-h post-isoprenaline (ISO, 50 mg/kg) treatment, one rat that developed atypical TS-like dysfunction (C and D), and one rat that retained normal morphology (E and F). The left ventricular ejection fraction (LVEF) of whole rats with TS-like dysfunction in the different groups in the present experiment was examined post-ISO (50 mg/kg) in the ISO group (panel G) ($P > 0.05$). Pretreatment with icariin prevented ISO stress-induced cardiac injury and showed mild dose dependence.

membranes were incubated with the following primary antibodies: TLR4 (Abcam, USA), NF-κB p65 (Santa Cruz Biotechnology, USA), MRP8 and MRP14 (Abcam, USA) and Actin (Servicebio, China). Thereafter, the membranes were incubated with a 1:5000 dilution of appropriate secondary HRP-conjugated antibodies for 1 h and visualized by enhanced chemiluminescence using an imaging system (Bio-Rad, Hercules, CA, USA). The data was normalized to the Actin content of the same sample.

2.8. Data and statistical analyses

IBM SPSS statistics software (version 25, USA) was used for standard statistical analysis of the data. Normal plots and the Kolmogorov–Smirnov test were used to verify the appropriateness of assuming a Gaussian distribution of the variables. All continuous variables were expressed as the mean ± standard deviation. Repeated measures ANOVA or the Mann–Whitney test was used to compare data between different groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Icariin decreased the morbidity of TS and the post isoprenaline mortality

The most notable result in the experiment was the difference in incidence of TS-like disease in the different treatment groups. Specifically, the rats in the Sha-group showed no obvious changes before and after the experiment. The lowest incidence of TS-like disease was found in the Ica-group, in which no rats died (Fig. 1 and Table 1A); however, four rats exhibited TS impairment. Conversely, eight rats developed TS-like pathology in the Con-group, and seven rats in the Met-group developed severe cardiac dysfunction; one case died in each group within 2 h after ISO administration (Table 1A). Three of the six rats developed TS-like impairment in the LPS-group, but no rats died.

In protocol 2, we investigated whether the cardioprotective effects of icariin were dose-dependent. As show in Table 2, the incidence of TS was identical in the M-group and H-group but was lower than that in the L-group. Specifically, four rats with TS-like pathological changes were noted in both the M-group and H-group, but no deaths occurred. Conversely, five rats developed severed cardiac dysfunction in the L-group, and one additional case died within 2 h.

3.2. Icariin alleviates isoprenaline stress-induced myocardial injury

At baseline, the body weights (BWs), blood pressure, and heart rates (HRs) were similar among the groups. The HRs diversification is a typical characteristic of rats post-ISO. As shown in Fig. 2A, the HRs of the rats increased remarkably after the ISO injection compared to those of the Sha-group at the different time points and then decreased gradually. Indeed, the average HR of the rats was lowest in the Ica-group. The mean HRs were similar in the Met-group and LPS-group and lower than that of the Con-group, although the differences were not significant. ISO obviously increased the blood pressure of the rats in the first hour,

Table 1A
Heart rate, cardiac function, morbidity and mortality of rats post isoprenaline 2 h and blood pressure 6 h.

	Sha-group (n = 6)	Con-group (n = 12)	Met-group (n = 12)	Ica-group (n = 12)	LPS-group (n = 6)	p-Value
HRs (bpm)	312.17 ± 39.42	504.64 ± 39.38***	497.91 ± 25.98***	469.75 ± 24.59***,#,&	462.67 ± 41.53***,#,&	< 0.001
SP (mmHg)	126.33 ± 15.35	84.50 ± 29.35**	88.25 ± 33.90**	95.33 ± 19.57*	95.17 ± 27.91*	0.046
DP (mmHg)	90.33 ± 17.42	61.67 ± 22.64*	63.75 ± 25.99*	70.33 ± 16.40	69.17 ± 21.87	0.11
MP (mmHg)	102.67 ± 15.92	69.00 ± 24.68**	71.75 ± 28.42**	77.83 ± 17.06*	77.17 ± 23.83*	0.067
LVEF (%)	68.5 ± 5.6	54.2 ± 27.3	55.5 ± 26.7	77.6 ± 21.1#,&	61.5 ± 24.5	0.275
CO (ml/min)	109.17 ± 18.26	138.50 ± 69.733	128.42 ± 76.62	174.25 ± 33.84*	127.33 ± 48.09	0.174
Morbidity	0	8	7	4	3	0.038
Mortality	0	1	1	0	0	0.728

All data are present with as mean ± SEM, HRs, heart rates; SP, systolic pressure; DP, diastolic pressure; MP, mean pressure; LVEF, left ventricular ejection fraction; CO, cardiac output; Morbidity, incidence of takotsubo syndrome; Mortality, the amount of rat death due to ISO injection.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ versus the Sha-group.
# $P < 0.05$ versus the Con-group.
& $P < 0.05$ versus the Met-group.

Table 1B
Body weight and myocardial enzyme in rats post isoprenaline 24 h in protocol 1.

	Sha-group (n = 6)	Con-group (n = 12)	Met-group (n = 12)	Ica-group (n = 12)	LPS-group (n = 6)	P-value
BWs (g)	331.33 ± 13.66	335.92 ± 10.39	334.00 ± 16.74	331.00 ± 17.01	334.83 ± 11.05	0.924
LDH (U/L)	184.17 ± 81.58	275.8 ± 258.31	429.56 ± 167.50*	402.33 ± 163.41*	713.00 ± 385.21***,#,&,$	0.002
CK (U/L)	258.33 ± 174.23	616.7 ± 589.36	1198.78 ± 912.06	581.75 ± 224.00	1530.67 ± 470.92**,#,$	0.01
CKMB (U/L)	246.83 ± 78.52	422.50 ± 292.79	415.78 ± 131.32	347.67 ± 102.73	594.83 ± 309.79**,$	0.056

All data are present with as mean ± SEM, BWs, body weights; LDH, lactate dehydrogenase; CK, creatine kinase; CKMB, creatine kinase isoenzyme.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ versus the Sha-group.
# $P < 0.05$ versus the Con-group.
& $P < 0.05$ versus the Met-group.
$ $P < 0.01$ versus the Ica-group.

Table 2
Body weight, cardiac function, morbidity and mortality of rats post isoprenaline 2 h and liver function and renal function in protocol 2.

	L-group (n = 12)	M-group (n = 12)	H-group (n = 12)	P-value
BWs (g)	333.58 ± 14.51	331.00 ± 17.01	329.42 ± 15.97	0.811
HRs (bpm)	481.44 ± 12.13	469.75 ± 24.6	485. ± 22.12	0.198
LVEF (%)	63.89 ± 26.35	79.33 ± 19.75	77.67 ± 22.56	0.269
CO (ml/min)	148.78 ± 56.06	174.25 ± 33.84	141.58 ± 25.96*	0.117
Morbidity	5	4	4	0.544
Mortality	1	0	0	0.368
BUN (μmol/L)	5.78 ± 0.48	5.64 ± 0.55	6.07 ± 0.82	0.879
SCr (mmol/L)	48.69 ± 5.83	52.20 ± 6.74	50.28 ± 6.42	0.509
ALT (U/L)	23.54 ± 5.95	26.98 ± 4.52	27.07 ± 12.54	0.717
AST (U/L)	37.04 ± 3.68	37.27 ± 8.56	38.98 ± 10.63	0.904

All data are present with as mean ± SEM, HR, heart rate; LVEF, left ventricular ejection fraction; CO, cardiac output; Morbidity, incidence of takotsubo syndrome; Mortality, the amount of rat death due to ISO injection; Scr, Serum creatinine; BUN, Urea nitrogen; AST, aspartate aminotransferase, ALT, alanine aminotransferase.
* $P < 0.05$ versus the M-group.

but a significant drop occurred within 6 h, as shown in Table 1A and Fig. 2(B and C). Both the mean systolic pressure (SP) and diastolic blood pressure (DP) were significantly lower in the ISO-treated rats than in the rats that did not receive ISO, whereas the values of the other four groups were similar, even for the rats preconditioned with metoprolol or icariin.

ISO served as a double-edged sword for the cardiac function of the rats in the experiment. As shown in Tables 1A and 1B, the cardiac output was calculated from the stroke volume and HR. The left ventricular ejection fraction (LVEF) of the rats increased greatly in the absence of TS but decreased remarkably when TS impairment appeared. When we compared the four groups with ISO administration, we found that the LVEF of the rats preconditioned with icariin was higher than that of the other three groups, whereas the measurements for the Con-group, Met-group and LPS-group were similar. In protocol 2, the cardioprotective effect of icariin exhibited a mild dose-dependent effect. As shown in Table 2, the average LVEF for both the middle and high doses was higher than that of the L-group dose, but no rats died during the experiment. Strangely, the LVEF in the higher dosage group was not better than that of the middle dosage group.

Furthermore, we compared all rats with TS-like impairment in the different groups and evaluated the LVEF with relevant statistical modeling, as shown in Fig. 1F. We found that pretreatment with icariin alleviated ISO-induced cardiac impairment and slightly improved the LVEF. However, this protective effect was weakened by preconditioning in combination with LPS.

3.3. Detection of myocardial enzymes and liver function and renal function

The myocardial enzymes increased acutely 24-h post-isoprenaline administration compared with those of the Sha-group in protocol 1. The myocardium was injured obviously after high-dosage ISO administration. As shown in Table 2, myocardial enzymes, including lactate dehydrogenase (LDH), creatine kinase (CK) and creatine kinase isoenzyme (CKMB), were increased in all ISO injection groups compared to those of the Sha-group. In particular, preconditioning with metoprolol and icariin preserved the myocardial impairment that manifested as lower myocardial enzyme levels than those of the Con-group, whereas these markers were higher in the LPS-group than in the Met-group and Ica-group. Furthermore, the Liver function, including Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT), as well as the Renal function, including Serum creatinine (Scr) and Urea nitrogen (BUN) were tested in in protocol 2 to investigate whether icariin potentially toxicity at high concentration. Table 2 suggests that the levels of AST and ALT increased slightly in the condition of higher dosage of icariin, and there was no difference within three groups about renal function.

3.4. Low myocardial lipid accumulation and fibrosis following icariin pretreatment

No obvious pathological myocardial fibrosis was observed in the Sha-group based on Masson's trichrome staining, but a certain increase was observed in the Con-group and Met-group; further, a mild increase was found in the rats preconditioned with icariin post-ISO, including those in the Ica-group and LPS-group (Fig. 3).

The acute myocardial injury induced by ISO was confirmed by myocardial tissue staining. The intramyocardial lipid contents exhibited obvious accumulation after ISO injection in the four different groups compared to that of the Sha-group, especially in the rats with TS-like injury (Fig. 4). The lipid contents were higher in the Con-group than in the other three groups. Similarly, the collective lipid volume was significantly lower in the rats with atypical TS impairment in the Ica-group and Met-group than in the Con-group rats and the rats with LPS preconditioning.

3.5. Effects of icariin on ROS and TBARS and SOD

Oxidative stress has been proven to promote cardiac dysfunction and cardiomyocyte hypertrophy. Studies have reported detection of accumulated ROS in TS patients by endocardial biopsy [6]. To determine the presence of increased oxidative stress in the present experiment, the ROS levels were tested by DHE. As shown in the results, the ROS levels post-ISO increased notably in both the Con-group and Met-group compared to those of the Sha-group, whereas pretreatment with icariin significantly reduced ROS accumulation. However, ROS production increased greatly following application of LPS and icariin together (Fig. 5).

In addition, the measurement of TBARS was performed to determine the total burden of ROS. As showed in results (Fig. 6A), the TBARS levels in Con-group and Met-group significantly higher than Sha-group, while icariin precondition attenuated the TBARS levels, however, combination of LPS markedly increased the amount of TBARS. SOD was evaluated because it is the primary endogenous anti-oxidant enzyme

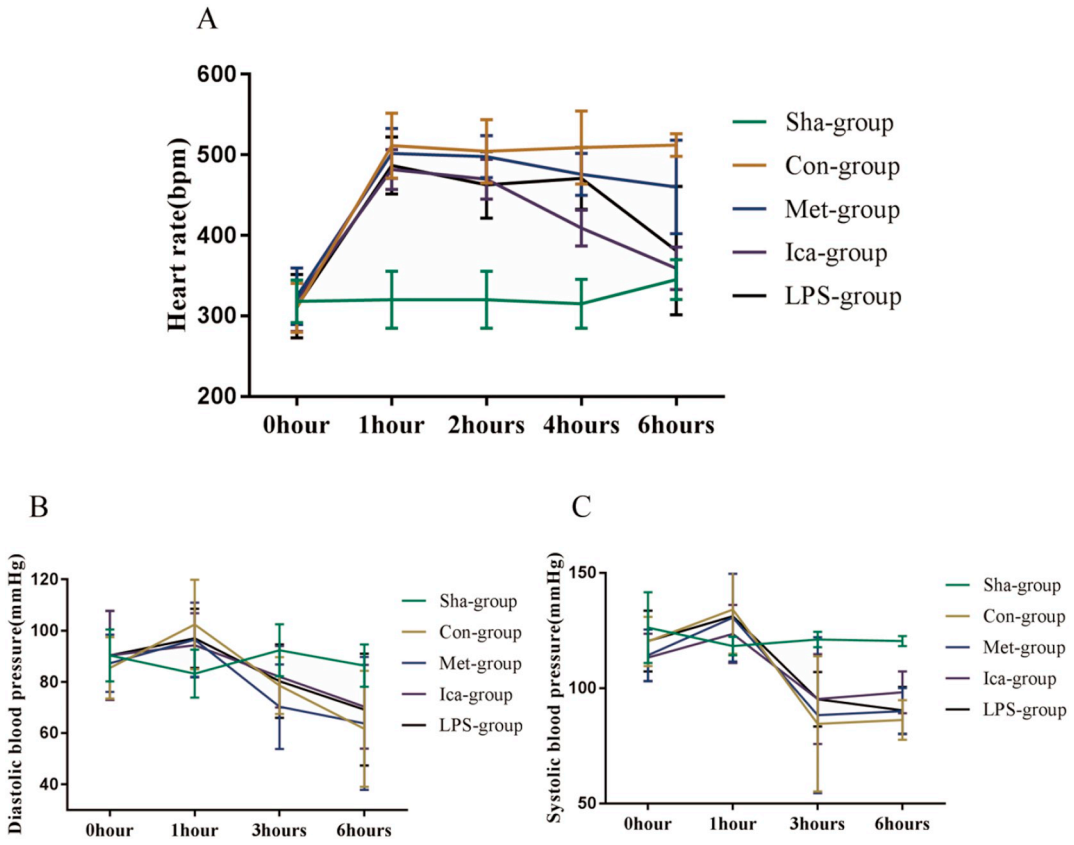

Fig. 2. The heart rates (HRs) (panel A) and blood pressure of the rats (panels B and C) in the different groups before and after isoprenaline (ISO, 50 mg/kg) administration at different time points. The HRs were similar at baseline among the five groups and increased obviously post-ISO treatment compared to those of the Sha-group (n = 6), peaked within 1 h, and then dropped gradually as recorded. The HRs of the rats in the Ica-group (n = 12) were significantly lower than those of the Con-group (n = 11) and Met-group (n = 11) rats at 2, 4 and 6 h ($P < 0.01$), whereas the HRs in the LPS-group (n = 6) were maintained at a stable level at 2 and 4 h and then dropped rapidly at 6 h. The average HRs in both the Ica-group and LPS-group decreased to the same level as the Sha-group at 6 h. Both the diastolic pressure (DP) and systolic pressure (SP) were increased slightly at 1-h post-ISO, but they dropped significantly at 6 h compared to the recorded data from the Sha-group. Specifically, the DP of the rats in the ISO administration groups dropped significantly ($P < 0.05$) and was maintained at same level at 6 h, although this value was significantly lower than that of the Sha-group ($P < 0.05$). The SP of the rats in all groups except the Sha-group dropped gradually after 1 h as recorded, and the SP in both the Con-group and Met-group was significantly lower than that of the Sha-group at 6-h post-ISO ($P < 0.05$).

that can neutralize free radicals and protect tissues from the effects of harmful free radical and other active oxygen species. However, no difference was found among the four isoprenaline treatment groups, which had significantly lower levels than the Sha-group (Fig. 6B).

3.6. Icariin normalized anti-oxidant elements and attenuated inflammatory gene expression in response to isoprenaline

To further investigate the variety of oxidative stress and anti-oxidative stress systems and the protective effect of icariin, the mRNA expression levels of myocardial anti-oxidant genes were tested by RT-PCR. Specifically, the gene expression of anti-oxidant elements, such as sod1 and cat, was decreased significantly post-ISO in both the Con-group and Met-group. However, the expression of these genes was maintained at a higher level in the Ica-group than in the Sha-group. By contrast, LPS administration significantly decreased both sod1 and cat expression (Fig. 6C and D).

Extensive studies have reported that accumulated ROS increases the production of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, which have also been found in TS patients and are thought to play important roles in the development of TS. Moreover, the release of excessive inflammatory factors and over-active inflammatory responses increase ROS production. Thus, selective proinflammatory factors were tested by RT-PCR. As exhibited in the results (Fig. 7H–J), the TNF-α, IL-1β and IL-6 levels were increased significantly in both the Con-group and Met-group compared to those of the Sha-group but were maintained at low levels in the rats preconditioned with icariin. By contrast, LPS administration increased the levels of pro-inflammatory factors, especially IL-1β and IL-6, although the TNF-α level was similar in these two groups with icariin preconditioning.

3.7. Icariin suppressed MRP8/MRP14 and TLR4/NF-κB signaling protein activation in the stress-induced TS-like rat model

To further investigate the mechanism underlying the cardioprotective effects of icariin in the development of TS in the rat model, TLR4 and downstream substrates were analyzed by Western blotting. LPS was

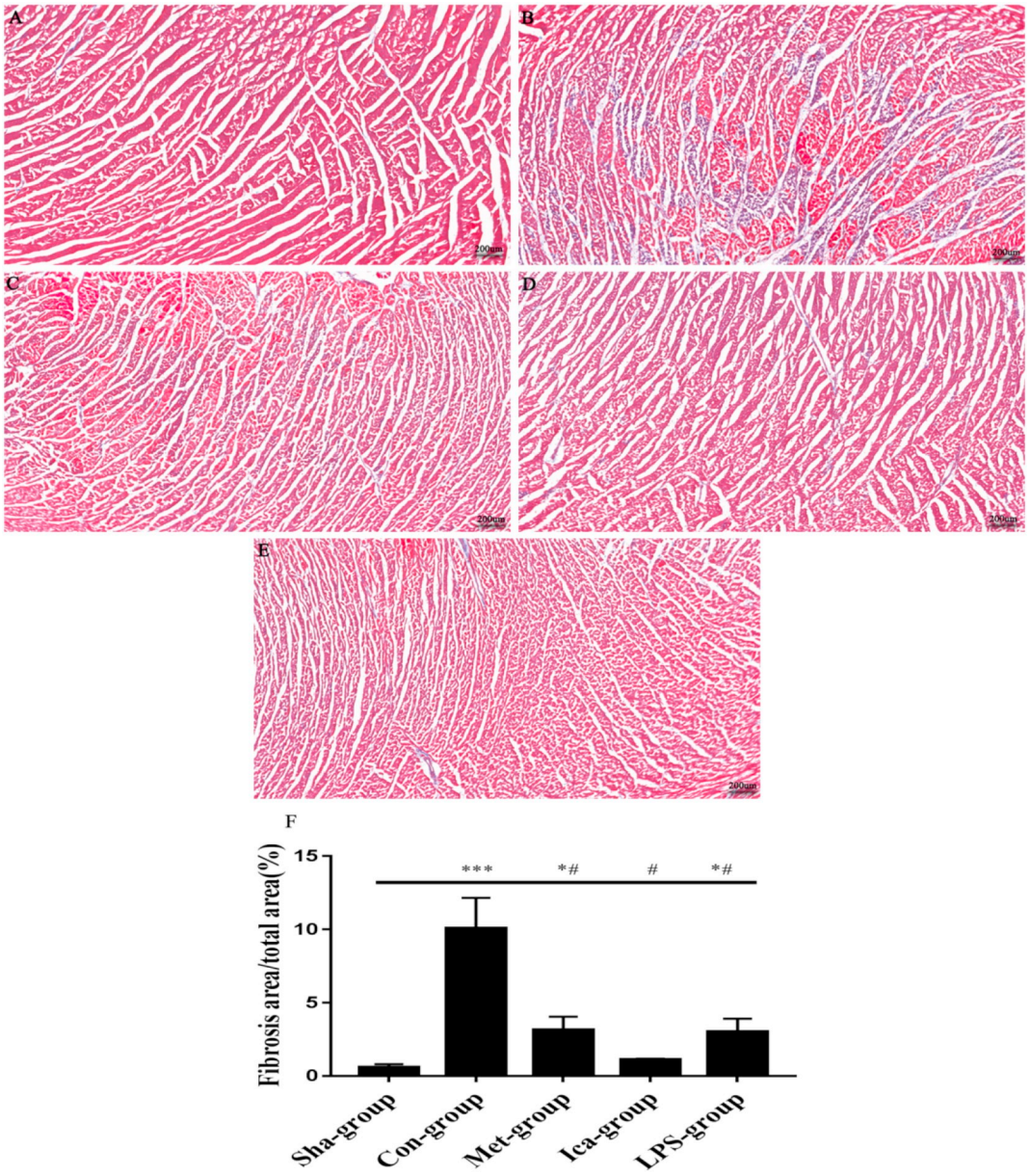

Fig. 3. Extent of cardiac fibrosis of the left ventricule at 24-h post-isoprenaline or saline administration (Masson's trichrome stain). Tissue from the apical regions of rats in the different groups. Rats with ISO injection show obvious fibrosis (B, n = 11) compared with that of tissues taken from the rats that received saline injection (A, n = 6). Preconditioning with metoprolol (C, n = 11) and icariin (D, n = 12) prevented ISO-induced tissue fibrosis, but LPS administration attenuated the protective effects of icariin (E, n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the Sha-group, $^{\#}P < 0.01$ versus the Con-group, $^{\&}P < 0.01$ versus the Met-group, $^{\$}P < 0.01$ versus the Ica-group.

selected as a TLR4 agonist in the study due to research documenting the ability of icariin to attenuate inflammatory action by inhibiting TLR4. The results showed that the level of phosphorylated TLR4 was significantly elevated in the ISO-treated groups compared to that of the Sha-group. This activation was inhibited greatly in the icariin pretreated rats (Fig. 7A–B), whereas this phenomenon was eliminated in the rats treated with LPS and icariin together. Furthermore, icariin reduced the phosphorylation and degradation of NF-κB in response to ISO administration and subsequently decreased the nuclear translocation and phosphorylated NF-κB p65 level (Fig. 7A–D), whereas LPS administration significantly increased NF-κB p65 expression. Finally, the alarmin family member myeloid-related protein-8 (MRP8) and -14 (MRP14), having been identified as vital endogenous proteins in modulation of inflammatory responses [29], were analyzed to explore the possible way of TLR4 inhibited by icariin. The results revealed that MRP8 and MRP14 expression were dramatically down-regulated at the protein levels in Ica-group compared with those of con-group and Sha-group, whereas they were up-regulated slightly in LPS-group.

4. Discussion

In the current study, we reported for the first time the

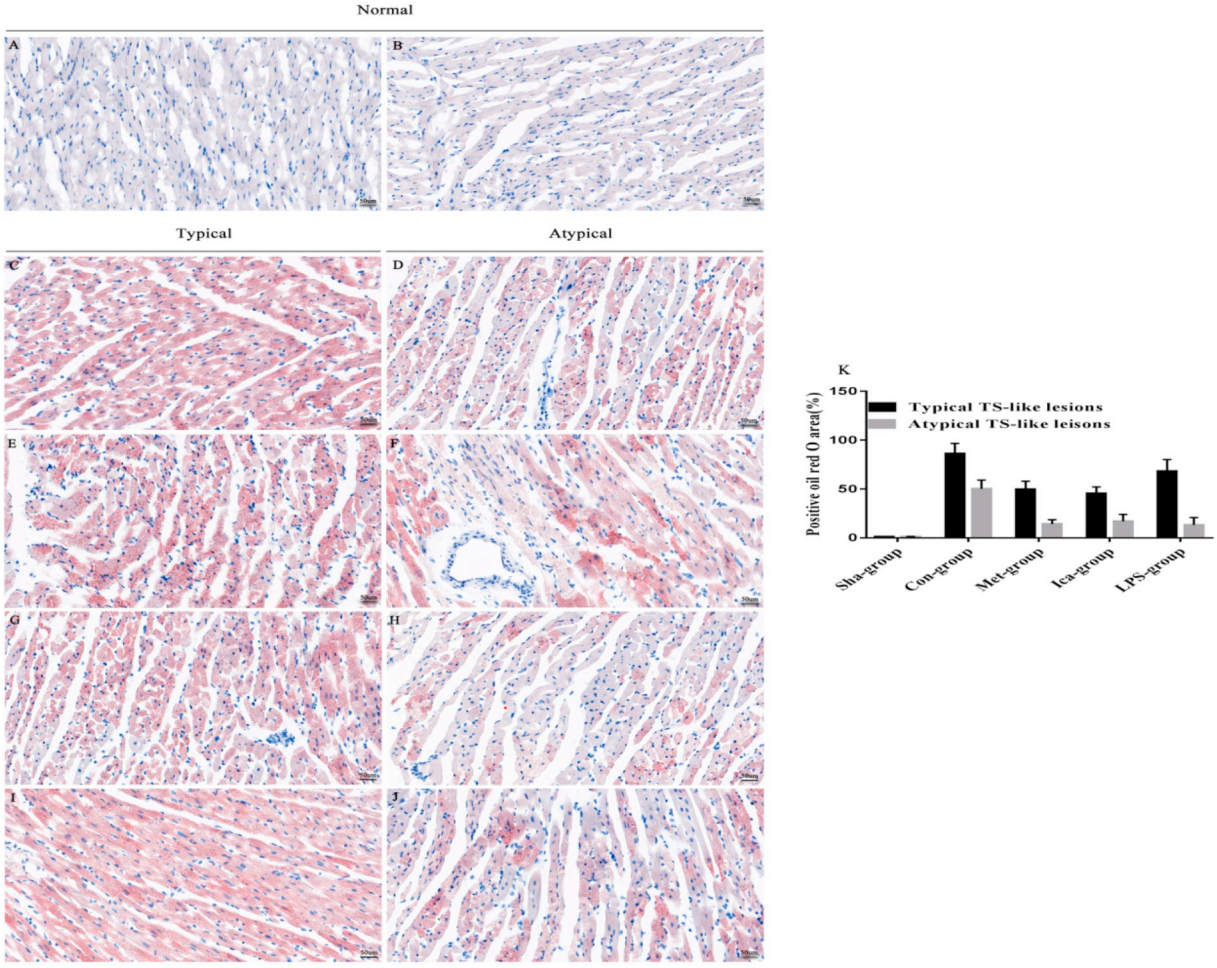

Fig. 4. Intracellular lipid accumulation in the akinesis region or apical segment at 24-h post-ISO (oil red O staining). (A and B, n = 6) Representative images were obtained from rats at the apical segment in the Sha-group with saline injection with nonballooning. (C and D, n = 8) Representative images of oil red O staining were obtained from rats that received only ISO injection. (E and F, n = 7) Tissues were obtained from rats that received with both metoprolol and ISO administration. (G and H, n = 4) Images of rats administered both icariin and ISO. (I and J, n = 3) Tissues were obtained from rats with ISO, icariin, and LPS administration. Intracellular lipid accumulation was much higher in the rats with typical TS-like disease who received only an ISO injection, was lower in the rats pretreated with metoprolol or icariin, and was slightly increased in the rats preconditioned with LPS. Quantification of intracellular lipids was higher in the atypical TS-like rats administered only ISO but was decreased obviously in the other three groups preconditioned with metoprolol or icariin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the Sha-group, $^{\#}P < 0.01$ versus the Con-group, $^{\&}P < 0.01$ versus the Met-group, $^{\$}P < 0.01$ versus the Ica-group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cardioprotective effects of icariin on an ISO-induced TS-like rat model. Mechanistically, icariin provides substantial concentration-dependent cardioprotective effects against ISO-induced stress in animal experiments, which may mainly be attributed to alleviating oxidative stress and reducing TLR4/NF-κB expression.

TS is common among patients with chest pain in the emergency room as well as in coronary care and intensive care unit patients with rapidly deteriorating heart function [30]. Studies reported that TS had defined mortality [31,32] and was considered an independent predictor of noncardiac death in the TS patient group [33]. Nevertheless, no guideline is available to provide adequate support for clinical decision-making at present. In the present study, we adopted a TS-like rat model to mimic clinical patients; this model is thought to be a suitable model of TS [34,35]. Icariin has been applied in the clinic and has been proven to be safe and effective [36]. Because myocardial adrenoceptor has been considered to play a key role in the pathogenesis of TS, metoprolol was selected as a control drug [37].

First, the incidence of TS-like cardiac dysfunction were investigated in different groups via echocardiography. Icariin, unlike metoprolol, exerted distinct positive effects on LV morphology and function, as evidenced by the morbidity and mortality of the rats. Then, the cardioprotective effects of icariin in a dose-dependent was confirmed in protocol 2. Accordingly, the protective effects were partly offset by LPS, which increased the TS-like incidence and mortality. By contrast, metoprolol had no effect on the incidence of stress-induced cardiac dysfunction, although substantial evidence proved that it could reduce the area of myocardial necrosis by selectively inhibiting the myocardial adrenergic receptor and sympathetic excitability from ischemia [38].

What is the actual mechanism involved in the cardioprotective effect? A number of studies have illustrated the benefits of icariin treatment for cardiovascular diseases, and evidence suggests that icariin may act on multiple targets, especially anti-oxidant stress. Generally, ROS is known to play a role as a double-edged sword in cellular activity. In particular, superphysiological levels of ROS are involved in the

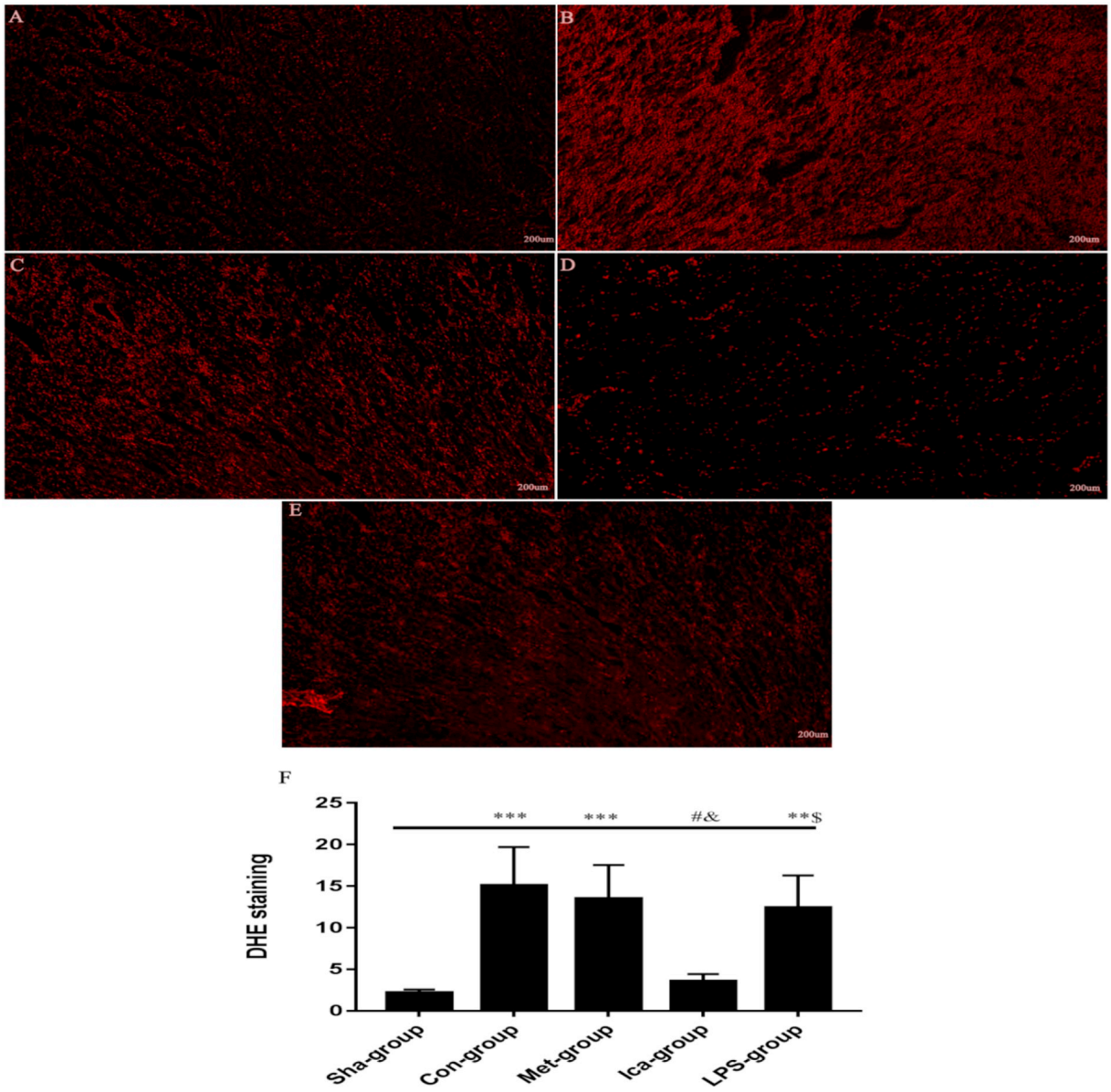

Fig. 5. Icariin significantly reduced the production of reactive oxygen species (ROS) induced by ISO injection. The high-dosage isoprenaline (ISO, 50 mg/kg) injection increased ROS accumulation obviously in the Con-group (B, n = 8) compared to that of the rats that received the saline injection in the Sha-group (A, n = 6) based on DHE analysis. Additionally, metoprolol had no effects on ROS production in the Met-group (C, n = 7). Surprisingly, icariin preconditioning notably reduced ROS production in the Ica-group (D, n = 4); however, LPS administration reversed the effects of icariin on ROS production by increasing ROS production in the LPS-group (E, n = 3). ROS production in the left ventricle (LV) sections was evaluated by DHE staining at 24-h post-ISO. $^{***}P < 0.001$, $^{**}P < 0.01$ versus the Sham-group, $^{\#}P < 0.01$ versus the Con-group; $^{\&}P < 0.01$ versus the Met-group; $^{\$}P < 0.01$ versus the Ica-group.

development of cardiac hypertrophy and heart failure, but these effects can be reversed by inhibiting oxidative stress [39]. The structure and sequence of icariin is similar to endogenous isoflavones that can promote ROS metabolism, including hydrogen peroxide and O_2^-. In agreement with other studies that icariin preconditioning decreased serum TBARS and ROS significantly in the ISO-injected rats. Furthermore, icariin upregulated the expression of anti-oxidant element genes, such as sod1 and cat, to maintain the balance between the oxidant and anti-oxidant systems.

Growing data suggest that accumulated ROS promotes pro-inflammatory cytokine generation and inflammatory cell release [40], which are tightly connected with cardiac dysfunction and progression from cardiac injury to failure [17,18]. Additionally, compelling evidence exists for the contribution of TLR4 participation [41]. In one mechanism, TLR4 is dependent on the activated transcription factors and triggers a series of inflammatory cascades. Specifically, TLR4 is exported to the plasma membrane, where it responds to its ligands, including the fusion protein from respiratory syncytial virus and heat shock protein, alters the composition and structure of the TLR4 ectodomain, and then activates the transcription factor NF-κB via the

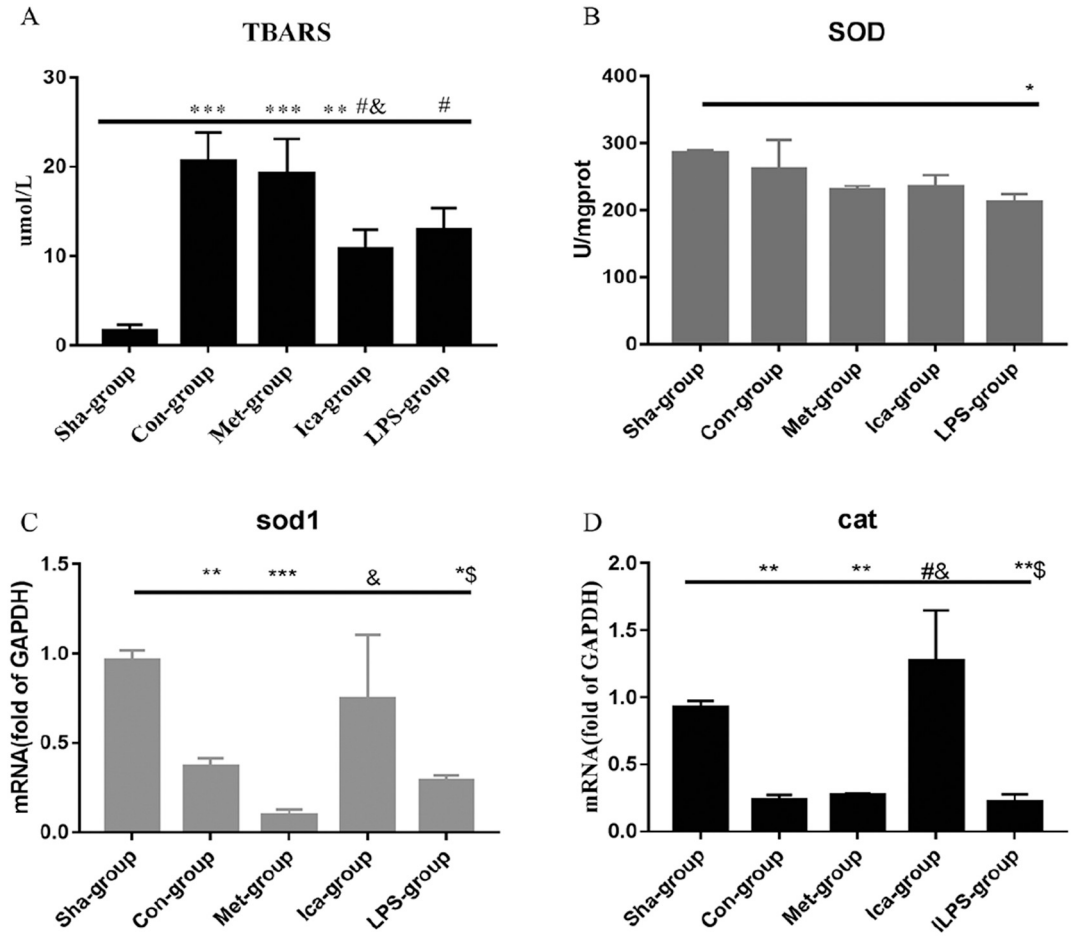

Fig. 6. Icariin reduces the levels of TBARS, but LPS combination increases the amount of TBARS (panel A). Nevertheless, the SOD activity was similar among all groups except for the LPS-group (panel B). Icariin sustains the expression of anti-oxidant genes following isoprenaline (ISO) injection. ISO injection significantly decreased both cat and sod1 mRNA expression in the Con-group and Met-group compared to that of the Sha-group as measured by RT-PCR (panel C and D). Preconditioning with icariin obviously increased the expression this element, whereas LPS administration reversed this phenomenon. The data are presented as the mean ± SE from two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the Sha-group, $^{\#}P < 0.01$ versus the Con-group, $^{\&}P < 0.01$ versus the Met-group, $^{\$}P < 0.01$ versus the Ica-group.

myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, thereby subsequently inducing the production of pro-inflammatory cytokines and interferons (IFNs), respectively. Icariin has been identified as having anti-inflammatory effects in previous studies, and these effects occur mainly by inhibiting activation of the TLR4 signaling pathway [42]. In the present study, we found that icariin pretreatment significantly decreased the activity of TLR4 and its downstream target proteins, including both total and p65 NF-κB, and decreasing the secretion of inflammatory factors and mediators. However, this effect was partially abolished by pretreatment with both icariin and LPS. Further research found that effects of icariin on TLR4 were primarily inhibiting upstream protein levels of MRP8/14, instead of acting on TLR4 directly [43]. While the major pathway activated by LPS is through TLR4/MD-2 complex formulation under the condition of CD14 and then triggers downstream pro-inflammation pathway [44]. These findings indicate that TLR4 plays a crucial role in positive regulation of the production of inflammatory cytokines in vivo and that the cardioprotective effects of icariin are mediated by suppression of the TLR4/NF-κB pathway.

5. Limitation

Takotsubo syndrome is a type of extremely complex acute cardiac syndrome, and a unifying mechanistic explanation is not available for this acute but rapidly reversible contractile dysfunction. Present study focused on the roles of ROS and inflammation in the development of TS, however, failed to elucidated the protective effect of icariin on the HRs of rats post-ISO. Furthermore, icariin exhibited obvious cardioprotection in the ISO stress-induced TS rat model but did not eliminate the complete impairment induced by ISO compared to the effects of isoflurane [34]. Finally, the Electron paramagnetic resonance (EPR), which is the gold standard to analysis ROS was not use in the experiment, is another flaw of this study. Therefore, more effort is needed to explore the mechanism of TS.

In summary, this report is the first to demonstrate the protective effect of icariin on the ISO stress-induced TS model. In addition, a possible mechanism was proposed for this protection involving the TLR4/NF-κB pathway and provided a probable method to prevent TS attacks.

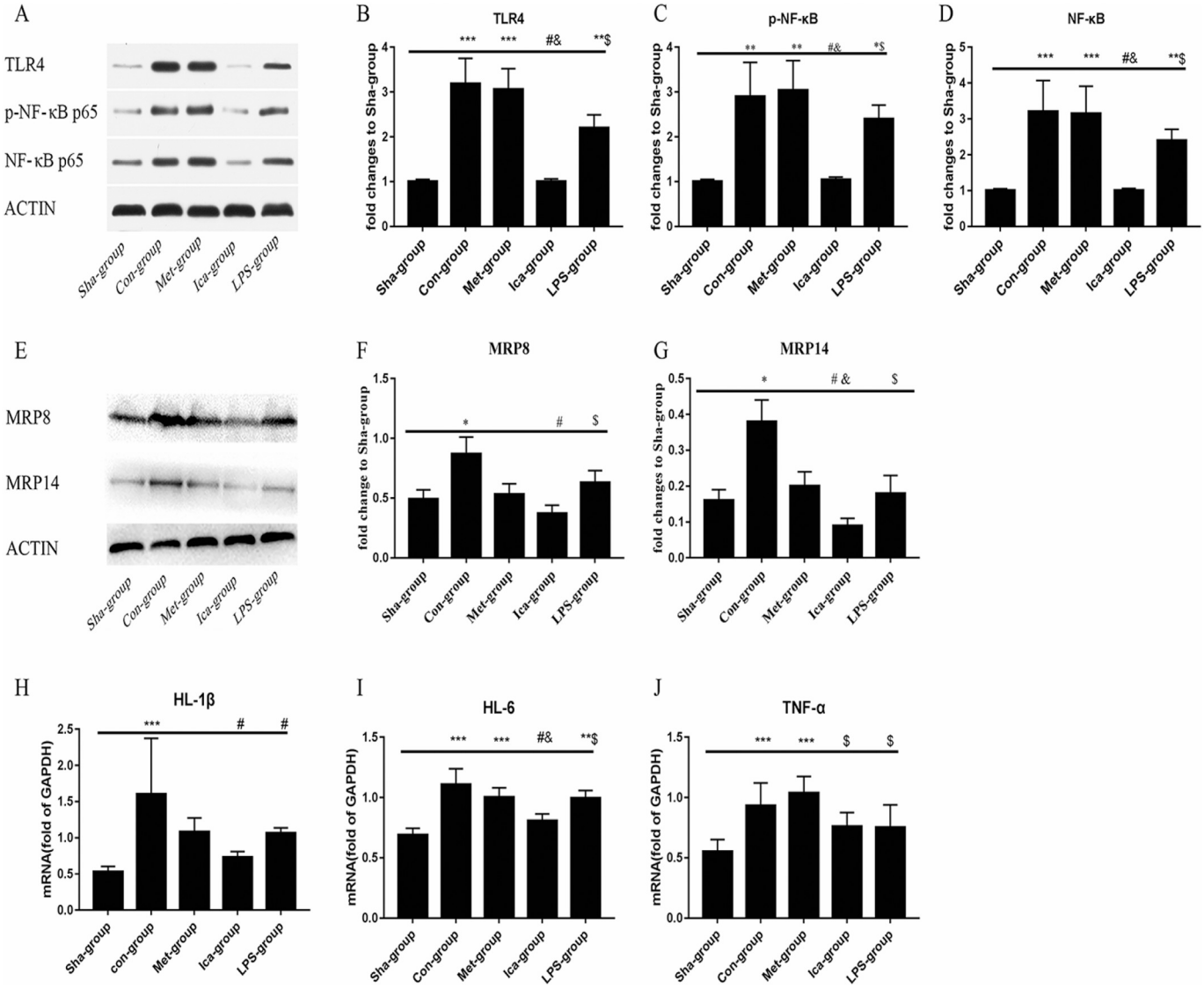

Fig. 7. Icariin suppresses the protein expression of Toll-like receptor (TLR)-4/nuclear factor kappa B (NF-κB) (panel A–D) and myeloid-related protein-8 (MRP8) and -14 (MRP14) (panel E–G). Accordingly, icariin decreases the mRNA expression of pro-inflammatory molecules in the isoprenaline (ISO)-induced cardiac acute impairment model based on real-time quantitative PCR (RT-PCR). Rats were treated with saline, ISO, ISO and metoprolol, ISO and icariin, ISO and icariin and LPS, and all rats were sacrificed at 24 h, the proteins level of TLR-4 and phospho-p65 and MRP8/14 were examined by Western blotting analysis. Pro-inflammatory molecules, including IL-1β, IL-6 and were determined by q-PCR. The data are presented as the mean ± SE from two independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the Sha-group, $^{\#}P < 0.01$ versus the Con-group, $^{\&}P < 0.01$ versus the Met-group, $^{\$}P < 0.01$ versus the Ica-group.

For peer review

Pertaining to claims relating to the content of this article will be borne by the authors. Chunlei Qi and Daxin Wang designed all the experiments and revised the paper. Yangzhen Shao, Xuesong Liu, Xueping Li performed the experiments, Chunlei Qi wrote the paper.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgement

This work was supported by grants from Jiangsu Provincial Science and technology support program (Social development, NO.BE2010697), “Six talent peaksproject” in Jiangsu Province (2014-SWYY-052), Jiangsu Provincial “333 Engineering” (BE2010697).

References

[1] A.B. Kevin, P. Abhiram, Stress-related cardiomyopathy syndromes, Circulation 118 (4) (2008) 397–409.
[2] M. Gianni, F. Dentali, A.M. Grandi, et al., Apical ballooning syndrome or takotsubo cardiomyopathy: a systematic review, Eur. Heart J. 27 (13) (2006) 1523–1529.
[3] O. Azrial, M. Shlomi, B. Roy, et al., Transient left ventricular apical ballooning (Tako-tsubo): the syndrome that mimics acute myocardial infarction, Isr. Med. Assoc. J. 6 (9) (2004) 550.
[4] H.M. Nef, M.L. Helge, E.S. Albrecht, Tako-tsubo cardiomyopathy (apical ballooning), Heart 93 (10) (2007) 1309–1315.
[5] I.S. Wittstein, D.R. Thieman, J.A. Lima, et al., Neurohumoral features of myocardial stunning due to sudden emotional stress, N. Engl. J. Med. 352 (6) (2005) 539–548 10.
[6] H.M. Nef, H. Möllmann, C. Troidl, et al., Expression profiling of cardiac genes in Tako-Tsubo cardiomyopathy:insight into a new cardiac entity, J. Mol. Cell. Cardiol. 44 (2008) 395–404.
[7] F. Santoro, M.D. Costantino, F. Guastafierro, et al., Inflammatory patterns in takotsubo cardiomyopathy and acute coronary syndrome: a propensity score matched analysis, Atherosclerosis 274 (2018) 157–161.
[8] S.P. Julie, J.A. Buckingham, S.J. Roebuck, et al., Topology of superoxide production from different sites in the mitochondrial electron transport chain, J. Biol. Chem. 277 (47) (2002) 44784–44790.
[9] A.A. Starkov, F. Gary, C. Christos, et al., Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species, J. Neurosci. 24 (36) (2004)

7779–7788.
[10] E.B. Tahara, F.D. Navarete, A.J. Kowaltowski, Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation, Free Radic. Biol. Med. 46 (9) (2009) 1283–1297.
[11] M.D. Brand, The sites and topology of mitochondrial superoxide production, Exp. Gerontol. 45 (7–8) (2010) 466–472.
[12] D.B. Sawyer, D.A. Siwik, L. Xiao, et al., Role of oxidative stress in myocardial hypertrophy and failure, J. Mol. Cell. Cardiol. 34 (4) (2002) 379–388.
[13] D.A.K. Eiki Takimoto, Role of oxidative stress in cardiac hypertrophy and remodeling, Hypertension 49 (2007) 241–248.
[14] L.L. Pan, X.H. Liu, Q.H. Gong, et al., S-Propargyl-cysteine (SPRC) attenuated lipopolysaccharide-induced inflammatory response in H9c2 cells involved in a hydrogen sulfide-dependent mechanism, Amino Acids 41 (1) (2011) 205–215.
[15] M. Dong, N. Hu, Y. Hua, et al., Chronic Akt activation attenuated lipopolysaccharide-induced cardiac dysfunction via Akt/GSK3β-dependent inhibition of apoptosis and ER stress, Biochim. Biophys. Acta 1832 (6) (2013) 848–863.
[16] Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila toll protein signals activation of adaptive immunity. Nature 1997; 388(6640):394–397.
[17] Y. Yang, J. Lv, S. Jiang, et al., The emerging role of toll-like receptor 4 in myocardial inflammation, Cell Death Dis. 7 (2016) e2234.
[18] S.B. Ong, S. Hernández-Reséndiz, G.E. Crespo-Avilan, et al., Inflammation following acute myocardial infarction: multiple players, dynamic roles, and novel therapeutic opportunities, Pharmacol. Ther. 186 (2018) 73–87.
[19] A. Ko odzi ska, K. Czarzasta, B. Szczepankiewicz, et al., Toll-like receptor expression and apoptosis morphological patterns in female rat hearts with takotsubo syndrome induced by isoprenaline, Life Sci. 199 (2018) 112–121.
[20] M. Ding, A. Pan, J.E. Manson, et al., Consumption of soy foods and isoflavones and risk of type 2 diabetes: a pooled analysis of three US cohorts, Eur. J. Clin. Nutr. 70 (12) (2016) 1381–1387.
[21] L. Gu, M.A. Kelm, J.F. Hammerstone, et al., Concentrations of proanthocyanidins in common foods and estimations of normal consumption, Eur. J. Clin. Nutr. 134 (3) (2004) 613–617.
[22] C.P. Bondonno, K.D. Croft, N. Ward, et al., Dietary flavonoids and nitrate: effects on nitric oxide and vascular function, Nutr. Rev. 134 (3) (2015) 613–617.
[23] Y. Hu, K. Liu, M. Yan, et al., Effects and mechanisms of icariin on atherosclerosis, Int. J. Clin. Exp. Med. 8 (3) (2015) 3585–3589.
[24] Z.Q. Liu, X.Y. Luo, Y.X. Sun, et al., The antioxidative effect of icariin in human erythrocytes against free-radical-induced haemolysis, J. Pharm. Pharmacol. 56 (12) (2004) 1557–1562.
[25] P.H. Ma, N.X. Ma, B.F. Ge, et al., Icariin attenuates hypoxia induced oxidative stress and apoptosis in osteoblasts and preserves their osteogenic differentiation potential in vitro, Cell Prolif. 47 (6) (2014) 527–539.
[26] H. Shao, J. Shen, M. Wang, et al., Icariin protects against titanium particle-induced osteolysis and inflammatory response in a mouse calvarial model, Biomaterials 60 (2015) 92–99.
[27] L. Kong, J. Liu, J. Wang, et al., Icariin inhibits TNF-α/IFN-γ induced inflammatory response via inhibition of the substance P and p38-MAPK signaling pathway in human keratinocytes, Int. Immunopharmacol. 29 (2) (2015) 401–407.
[28] M.F. Walter, R.F. Jacob, B. Jeffers, et al., Serum levels of thiobarbituric acid reactive substances predict cardiovascular events in patients with stable coronary artery disease: a longitudinal analysis of the PREVENT study, J. Am. Coll. Cardiol. 44 (10) (2004) 1996–2002.
[29] A. Schiopu, O.S. Cotoi, S100A8 and S100A9: DAMPs at the crossroads between innate immunity, traditional risk factors, and cardiovascular disease, Mediat. Inflamm. 2013 (2013) 828354.
[30] R. Shiomura, S. Nakamura, H. Takano, et al., Impact of brain natriuretic peptide, calcium channel blockers, and body mass index on recovery time from left ventricular systolic dysfunction in patients with takotsubo cardiomyopathy, Am. J. Cardiol. 116 (4) (2015) 515–519.
[31] T. Stiermaier, C. Moeller, K. Oehler, S. Desch, et al., Long-term excess mortality in takotsubo cardiomyopathy: predictors, causes and clinical consequences, Eur. J. Heart Fail. 18 (6) (2016) 650–656.
[32] K. Singh, K. Carson, R. Shah, et al., Meta-analysis of clinical correlates of acute mortality in takotsubo cardiomyopathy, Am. J. Cardiol. 113 (8) (2014) 1420–1428.
[33] M. Girardey, L. Jesel, U. Campia, et al., Impact of malignancies in the early and late time course of takotsubo cardiomyopathy, Circ. J. 80 (10) (2016) 2192–2198.
[34] B. Redfors, J. Oras, Y. Shao, et al., Cardioprotective effects of isoflurane in a rat model of stress-induced cardiomyopathy (takotsubo), Int. J. Cardiol. 176 (3) (2014) 815–821.
[35] Y. Shao, B. Redfors, M. Scharin Täng, et al., Novel rat model reveals important roles of β-adrenoreceptors in stress-induced cardiomyopathy, Int. J. Cardiol. 168 (3) (2013) 1943–1950.
[36] C. Li, Q. Li, Q. Mei, T. Lu, Pharmacological effects and pharmacokinetic properties of icariin, the major bioactive component in Herba Epimedii, Life Sci. 126 (2015) 57–68.
[37] A.R. Lyon, P.S. Rees, S. Prasad, et al., Stress (Takotsubo) cardiomyopathy-a novel pathophysiological hypothesis to explain catecholamine-induced acute myocardial stunning, Nat. Clin. Pract. Cardiovasc. Med. 5 (1) (2008) 22–29.
[38] A.S. Maisel, Beneficial effects of metoprolol treatment in congestive heart failure. Reversal of sympathetic-induced alterations of immunologic function, Circulation 90 (4) (1994) 1774–1780.
[39] N.R. Madamanchi, M.S. Runge, Redox signaling in cardiovascular health and disease, Free Radic. Biol. Med. 61 (2013) 473–501.
[40] K.A. Kaminski, T.A. Bonda, J. Korecki, W.J. Musial, Oxidative stress and neutrophil activation-the two keystones of ischemia/reperfusion injury, Int. J. Cardiol. 86 (1) (2002) 41–59.
[41] D. Fairweather, S. Yusung, S. Frisancho, et al., IL-12 receptor beta 1 and toll-like receptor 4 increase IL-1 beta- and IL-18-associated myocarditis and coxsackievirus replication, J. Immunol. 170 (9) (2003) 4731–4737.
[42] C.Q. Xu, B.J. Liu, J.F. Wu, et al., Icariin attenuates LPS-induced acute inflammatory responses: involvement of PI3K/Akt and NF-kappaB signaling pathway, Eur. J. Pharmacol. 642 (1–3) (2010) 146–153.
[43] J. Zhou, J. Wu, X. Chen, et al., Icariin and its derivative, ICT, exert anti-inflammatory, anti-tumor effects, and modulate myeloid derived suppressive cells (MDSCs) functions, Int. Immunopharmacol. 11 (7) (2011) 890–898.
[44] M. Rahimifard, F. Maqbool, S. Moeini-Nodeh, et al., Targeting the TLR4 signaling pathway by polyphenols: a novel therapeutic strategy for neuroinflammation, Ageing Res. Rev. 36 (7) (2017) 11–19.