



Qishen Yiqi dropping pills improve isoproterenol-induced cardiomyocyte hypertrophy by regulating X-inactive specific transcript (XIST) expression in rats

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Background: This study aimed to explore the potential mechanism of Qishen Yiqi dropping pills (QYDPs) in the treatment of chronic heart failure (CHF) by regulating the expression of lncRNAs during CHF.

Methods: Differences in the expression of the long non-coding RNA (lncRNA), X-inactive specific transcript (XIST), in an isoproterenol (ISO)-induced cardiomyocyte hypertrophy model treated with QYDPs was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). A cell counting kit-8 (CCK8) assay, flow cytometry (FCM), and enzyme linked immunosorbent assay (ELISA) were used to analyze the protective effects of QYDPs on the proliferation rate, apoptosis, myocardial enzyme, oxidative stress, and inflammation of cardiomyocytes, as well as the molecular mechanism of XIST.

Results: Our results showed that in the ISO-induced cardiomyocyte hypertrophy model, XIST expression and apoptosis were increased, the cell proliferation rate was decreased, and myocardial enzyme levels increased [i.e., increased lactate dehydrogenase (LDH) and creatine kinase (CK) levels]. Furthermore, cellular oxidative stress [i.e., increased malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD) levels] and inflammatory response [i.e., increased interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α protein secretion] were also promoted. QYDP treatment effectively mitigated the effects of ISO induction. Subsequently, we found that suppressing XIST expression reversed the effect of ISO induction, whereas overexpression (ov) of XIST enhanced the effect of ISO induction. Finally, this study confirmed that QYDP treatment improved the ISO-induced decrease in proliferation, apoptosis, and promotion of oxidative stress and inflammatory response in cardiomyocytes, whereas ov of XIST partially negated the effect of QYDPs.

Conclusions: QYDPs protected H9c2 cells from ISO-induced damage by downregulating XIST expression.

Keywords: Long non-coding RNA X-inactive specific transcript (lncRNA XIST); H9c2; chronic heart failure (CHF); oxidative stress; inflammation

Submitted Apr 02, 2022. Accepted for publication Jun 17, 2022.

doi: 10.21037/jtd-22-606

View this article at: <https://dx.doi.org/10.21037/jtd-22-606>

Introduction

Despite significant advances in diagnosis and treatment over the last 20 years, including the development of angiotensin receptor blockers (1,2) and left ventricular assist devices (3), chronic heart failure (CHF) remains a leading cause of death (4). Numerous studies have attempted to determine the etiology of and treatment for CHF by analyzing its underlying pathological mechanisms (5-7). The use of isoproterenol (ISO) in rat cardiomyocytes (H9c2) to construct a cardiac hypertrophy model, which can effectively promote cell apoptosis and the levels of cardiac enzymes such as lactate dehydrogenase (LDH) and creatine kinase (CK), and reduce cell growth (8-10), is a common means of studying the molecular mechanisms of CHF *in vitro*.

Traditional Chinese medicine (TCM) for the treatment of CHF has the advantages of low-toxicity side effects and improved quality of life (11,12). Qishen Yiqi dropping pills (QYDPs) are composed of *Astragalus membranaceus*, *Salvia miltiorrhiza*, notoginseng, and *Lignum Dalbergiae odoriferae* oil, which are widely used in China as drugs to combat cardiovascular diseases (13). However, similar to many other TCMs, such as *Panax notoginseng* (*P. notoginseng*) saponins (14,15) and *Salvia miltiorrhiza* (16), the mechanism of action of QYDPs remains unclear.

The dysregulation of long non-coding RNAs (lncRNAs) is closely related to the progression of CHF (17,18). Li *et al.* (19) found that overexpression (ov) of the lncRNA, X-inactive specific transcript (XIST), was detrimental to the treatment of hypoxia/reoxygenation-induced rat cardiomyocyte (H9c2) injury. In addition, the expression of XIST is closely related to oxidative stress and inflammation (20,21). Therefore, we speculated that XIST is involved in the process of CHF; however, its role and potential underlying mechanism remain unclear.

New evidence has suggested that oxidative stress (22) and inflammation (23) are closely associated with the development and progression of CHF. QYDPs exert excellent anti-inflammatory (24) and cardiotoxic effects (13). In this study, by regulating the expression of the lncRNA XIST, we explored the molecular mechanism through which QYDP protects H9c2 cells from ISO-induced immunity, lncRNA XIST as a new therapeutic target provided a theoretical basis for the treatment of CHF based on pharmacology and molecular biology. We present the following article in accordance with the MDAR reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-606/rc>).

Methods

Cell culture and transfection

H9c2 rat cardiomyocyte cells from the American Type Culture Collection (Manassas, VA, USA) were stored in Dulbecco's modified Eagle's medium [37 °C, 5% carbon dioxide (CO₂)] containing 10% fetal bovine serum (FBS). Once the H9c2 cells reached 60–80% confluency, they were transfected with ov or small interfering (si) RNA XIST (100 nM) and their negative controls using Lipofectamine[®] 2000 (Invitrogen, Thermo Fisher Scientific, Inc., MA, USA) for 4 h. The H9c2 cells were pretreated with different concentrations of QYDPs (0, 10, 50, 100, 250, and 500 µg/mL) and then exposed to ISO (80 µM) for 48 h, as described in a previous study (25). XIST was synthesized by GenePharma Biotechnology Co., Ltd. (Shanghai, China), and the si-XIST sequences are listed in *Table 1*.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay

Total RNA was extracted from H9c2 cells using TRIzol reagent (Invitrogen). After centrifugation (4 °C; 10 min) at 13,000 ×g, the supernatant was adsorbed and discarded, and 10 µL diethyl pyrocarbonate (DEPC; Invitrogen) water was added to the precipitate. Complementary DNA was prepared by reverse transcription using the PrimeScript RT kit (Takara Bio, Japan). The SYBR[®] Premix Ex Taq[™] II kit (Takara) was used for RT-qPCR analysis using the Applied Biosystems[®] 7500 Real-Time PCR system (California, USA). PCR experiments were performed under the following conditions: 95 °C for 10 min, 55 °C for 2 min, and 72 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 32 s. The primer pair sequences are listed in *Table 2*. The XIST expression level was normalized to that of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and calculated using the 2^{-ΔΔCt} method (26).

Proliferation assay

Cell counting kit-8 (CCK8) reagent (Solarbio, Beijing, China; 10 µL) was added to 96-well plates (containing H9c2 at 4×10³ cells) at 0, 24, 48, and 72 h. After 60 min of incubation in the dark at 25 °C, the absorbance was measured at 490 nm using an enzyme-labeled instrument (Thermo Fisher Scientific).

Table 1 Sequences of the three siRNAs targeting XIST

siRNA symbol	Forward oligonucleotide 5'-3'	Reverse oligonucleotide 5'-3'
si-XIST-1	GAUUGUUAGUGUGAAUAAUUU	AUUAAUUCACACUAACAAUCAU
si-XIST-2	GCACGAUCAAGAAAUGUAAAC	UUACAUUUUCUUGAUCGUGCUG
si-XIST-3	GGAGAGAUGUGCCGAAUAAGA	UUAUUCGGCACAUCUCUCCUU
si-NC	UUCUCGGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

siRNA, small interfering RNA; XIST, X-inactive specific transcript; NC, negative control.

Table 2 Primer sequences used in this study

Gene symbol	Forward primer 5'-3'	Reverse primer 5'-3'
XIST	TTGAGCTGGCTCGACAGAATG	GGTCTTAGACGTCCCAGGC
GAPDH	TGGGGCCAAAAGGGTCATCA	GCAGGATGCATTGCTGACAA

GAPDH was used to normalize XIST expression. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; XIST, X-inactive specific transcript.

Apoptosis assay

H9c2 cells were washed with phosphate-buffered saline (PBS) and then incubated with 5 μ L annexin V-fluorescein isothiocyanate (FITC) for 15 min. Next, 5 μ L propidium iodide (PI) was added for another 5 min. Flow cytometry (FCM; BD Biosciences, CA, USA) was used to analyze cell apoptosis. The cells were incubated in the dark at 4 $^{\circ}$ C.

Reactive oxygen species (ROS) measurement by FCM

H9c2 cells (1×10^5) were incubated (37 $^{\circ}$ C) with 1.0 μ M 2,7-dichlorodihydrofluorescein diacetate. After 15 min, PRKs were then washed twice with PBS and analyzed by FCM to detect ROS using a 488-nm laser for excitation and a 535-nm laser for detection.

Enzyme-linked immunosorbent assay (ELISA)

The cell supernatant of each subgroup was collected to analyze the levels of LDH, CK, malondialdehyde (MDA), superoxide dismutase (SOD), and the inflammatory factors interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α . The following kits were used according to the manufacturer's instructions: LDH activity assay kit (Solarbio), CK activity assay kit (Solarbio), micro MDA assay kit (Solarbio), SOD activity detection kit (Solarbio), glutathione (GSH) activity detection kit (Solarbio), IL-1 beta ELISA kit (R&D Systems, Minneapolis, USA), IL-6 Quantikine ELISA kit (R&D Systems), and TNF- α Quantikine ELISA Kit (R&D Systems).

Statistical analysis

Data are presented as the mean \pm standard deviation from triplicate evaluations. Statistical analysis was performed using one-way analysis of variance and Bonferroni post hoc tests. The Student's *t*-test was used for independent two-group analyses. Differences were considered significant at $P < 0.05$.

Results

QYDP regulates H9c2 proliferation and apoptosis via XIST expression

The CCK8 assay showed that the proliferation of H9c2 cells decreased after pretreatment with 250 and 500 μ g/mL QYDPs for 4 h (Figure 1A). Our results indicated that the proliferation of H9c2 cells might be affected by pretreatment with QYDPs at a concentration greater than 250 μ g/mL (4 h). To prevent the interference of excessive QYDPs in the experiment, we selected the QYDP concentration of 100 μ g/mL (4 h) as the experimental concentration for subsequent analyses of the molecular mechanisms.

Next, ISO-induced H9c2 cells were used to construct an apoptosis model. RT-qPCR, CCK8 assay, and FCM results showed that ISO induction in H9c2 cells led to upregulated XIST expression, a decreased cell proliferation rate, as well as increased apoptosis and ROS levels (Figure 1B-1E). The effect of ISO induction was reduced after QYDP treatment in a concentration-dependent manner (0, 10, 50, and 100 μ g/mL).

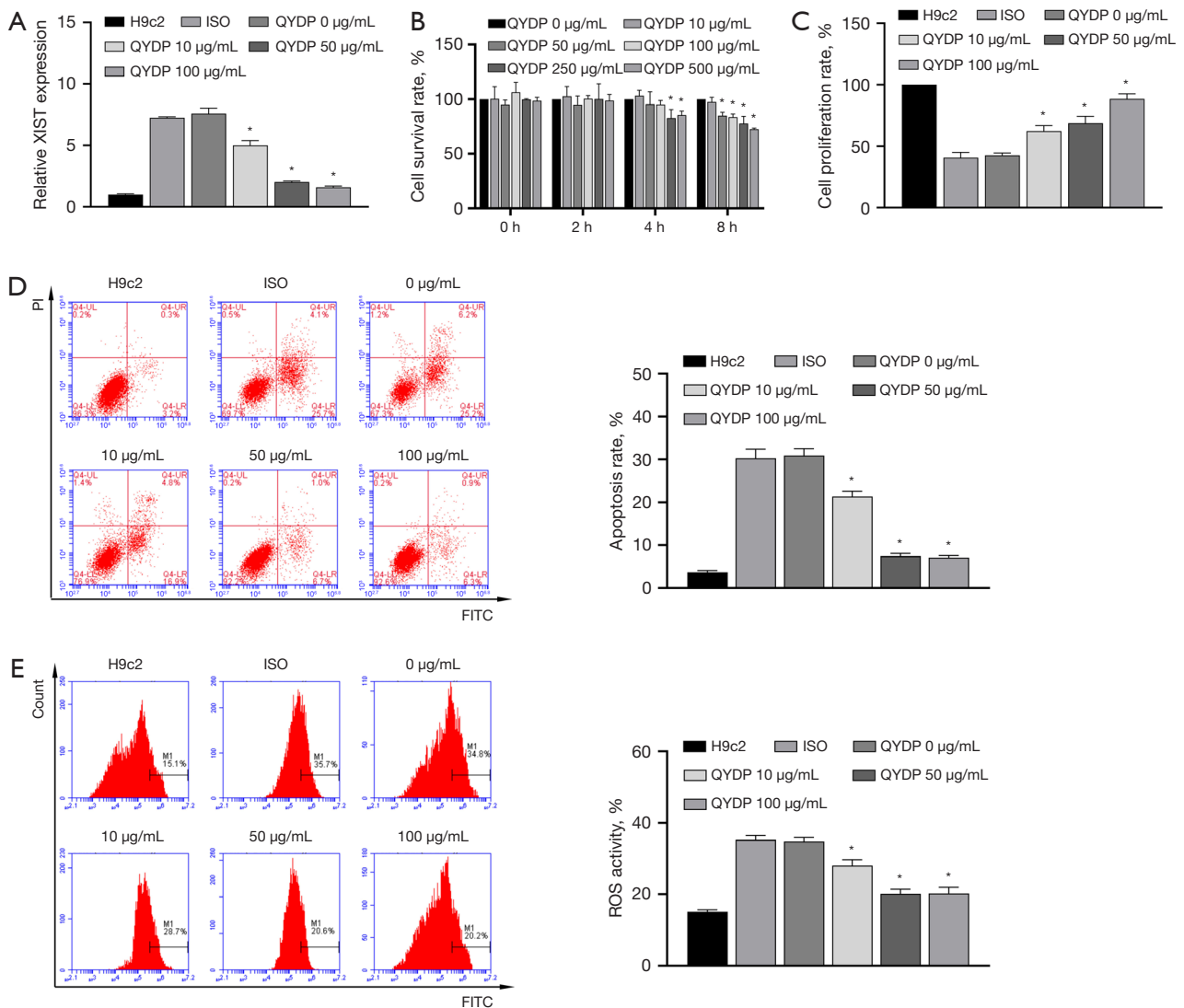


Figure 1 Effect of QYDPs on the expression of lncRNA, H9c2 cell proliferation and apoptosis, and ROS levels. (A) RT-qPCR analysis was performed to analyze the effect of ISO and QYDP on the expression of lncRNA in H9c2 cells. (B) A CCK8 assay was performed to assess the effect of gradient concentrations of QYDPs on the proliferation rate of H9c2 cells. (C) CCK8 assay was performed to analyze the effect of ISO and QYDP on the proliferation rate of H9c2 cells. (D,E) FCM was performed to analyze the effect of ISO and QYDP on apoptosis (D) and ROS levels (E) of H9c2 cells. * $P < 0.05$. XIST, X-inactive specific transcript; ISO, isoproterenol; QYDP, Qishen Yiqi dropping pills; PI, propidium iodide; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; lncRNA, long non-coding RNA; CCK8, cell counting kit-8; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ISO, isoproterenol.

QYDP treatment reduced ISO-induced oxidative stress and inflammation. The ELISA results showed that ISO induced an increase in the levels of myocardial enzymes [increased LDH and CK levels (Figure 2A,2B)], oxidative stress [increased MDA level (Figure 2C), and decreased SOD and GSH levels (Figure 2D,2E)] and inflammatory response [increased IL-1 β , IL-6, and TNF- α protein secretion

(Figure 2F-2H)] in H9c2 cells, which were inhibited by QYDP treatment.

Role and potential mechanism of XIST in the H9c2 cell apoptosis model

To further explore the mechanism of action of XIST,

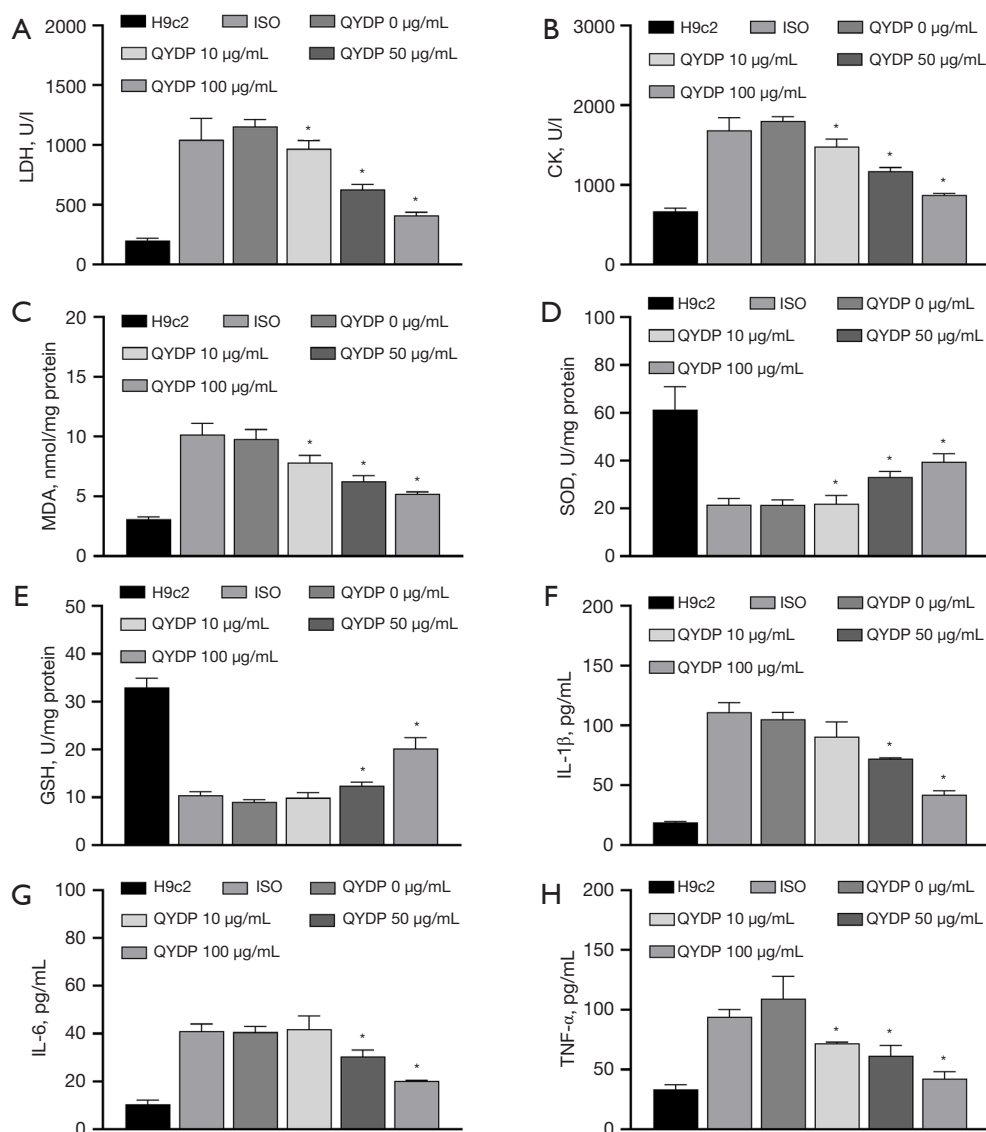


Figure 2 Effect of QYDPs on ISO-induced oxidative stress and inflammation in H9c2 cells. (A,B) ELISA was performed to analyze the effect of QYDPs on the levels of ISO-induced myocardial enzyme-related factors: LDH (A) and CK (B). (C-E) ELISA was performed to analyze the effect of QYDPs on the levels of ISO-induced oxidative stress-related factors: MDA (C), SOD (D), and GSH (E). (F-H) ELISA was performed to analyze the effect of QYDP on the levels of ISO-induced inflammation-related factors: IL-1 β (F), IL-6 (G), and TNF- α (H). * $P < 0.05$. LDH, lactate dehydrogenase; ISO, isoproterenol; QYDP, Qishen Yiqi Dropping Pills; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay.

we constructed an ov-XIST plasmid and three siRNAs targeting XIST and then transfected them into H9c2 cells. RT-qPCR results showed that XIST expression was increased in the ov-XIST transfection group and decreased in the three si-XIST transfection groups, indicating the successful construction of ov-XIST and si-XIST (Figure 3A).

Notably, si-XIST-1 showed the best inhibition efficiency and was thus selected as the antagonist of XIST (si-XIST) in subsequent experiments.

We transfected ov-XIST and si-XIST into ISO-induced H9c2 cells. The results of CCK8, FCM, and ELISA showed that ISO-induced inhibition of the proliferation rate of H9c2

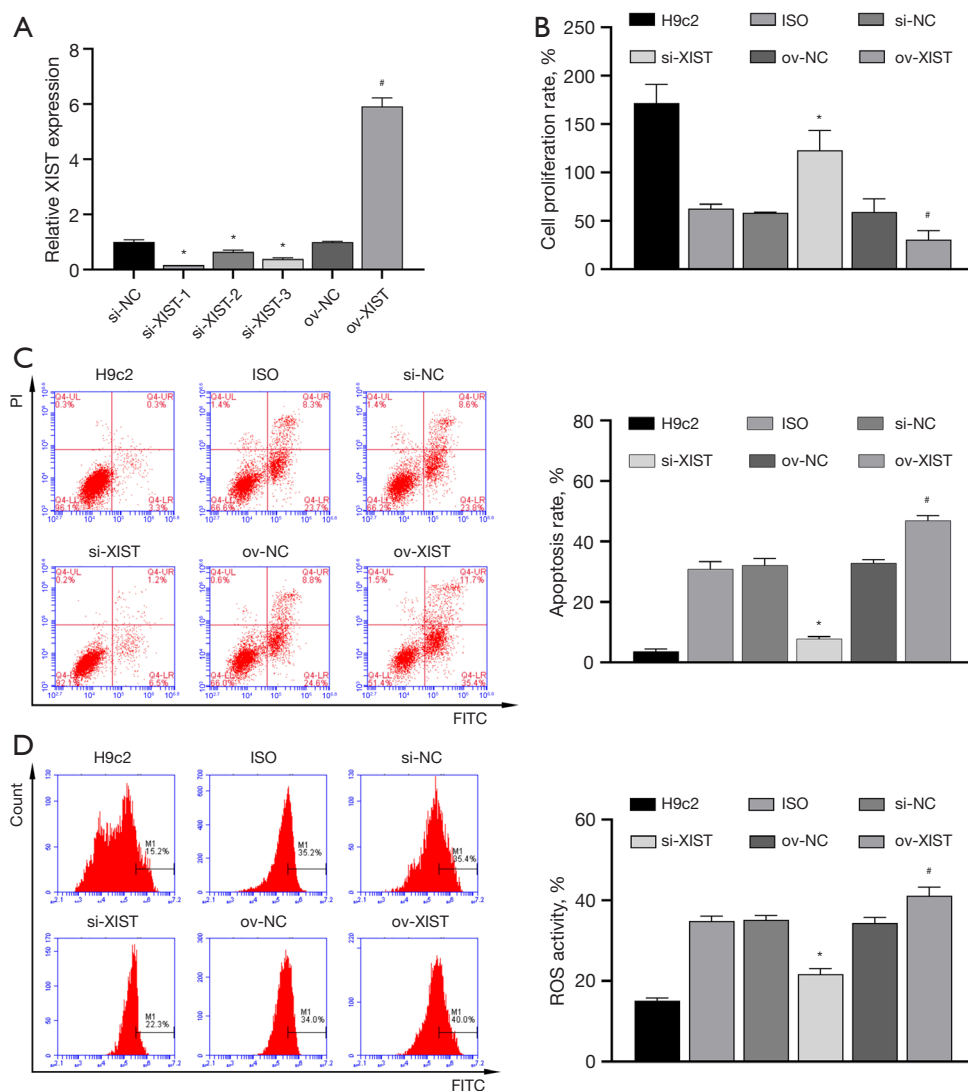


Figure 3 LncRNA reversed the effect of ISO induction in H9c2 cells. (A) RT-qPCR analysis of the effectiveness of over-expressing lncRNA plasmid and si-lncRNA. (B) A CCK8 assay was performed to assess the effect of lncRNA on the proliferation rate of H9c2 cells induced by ISO. (C,D) FCM was performed to analyze the impact of lncRNA on ISO-induced apoptosis (C) and ROS levels (D) of H9c2 cells. *, si-XIST vs. si-NC, $P < 0.05$; #, ov-XIST vs. ov-NC, $P < 0.05$. XIST, X-inactive specific transcript; si, small interfering; NC, negative control; ov, overexpression; ISO, isoproterenol; PI, propidium iodide; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction; CCK8, cell counting kit-8; FCM, flow cytometry.

cells (Figure 3B) and promotion of apoptosis (Figure 3C), ROS levels (Figure 3D), myocardial enzyme levels [increased LDH and CK levels (Figure 4A,4B)], oxidative stress [increased MDA level (Figure 4C), decreased SOD and GSH level (Figure 4D,4E)], and inflammatory reactions [increased secretion of IL-1 β , IL-6, and TNF- α proteins (Figure 4F-4H)] were all reversed by si-XIST. However, the

effect of QYDPs on the improvement of ISO induction in H9c2 cells was reversed by XIST ov.

To investigate the association between XIST expression and QYDPs, we added QYDP (100 $\mu\text{g/mL}$) to the overexpressed XIST transfection group. The CCK8, FCM, and ELISA results showed that QYDP treatment improved the inhibition of ISO-induced proliferation, apoptosis, myocardial enzymes,

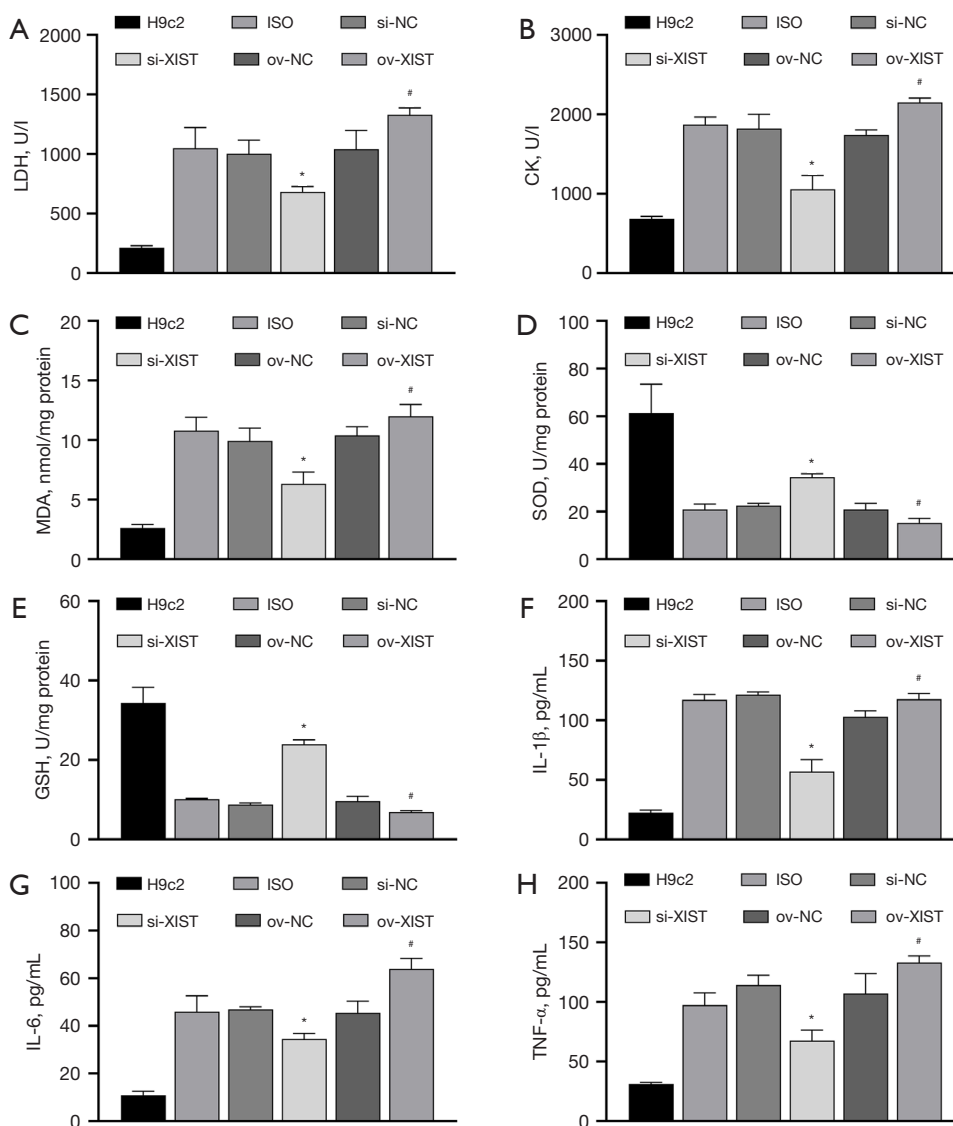


Figure 4 (A,B) ELISA was performed to analyze the effect of lncRNA on the levels of myocardial enzyme-related factors LDH (A) and CK (B) in ISO-induced H9c2 cells. (C-E) ELISA was performed to analyze the effect of lncRNA on the levels of oxidative stress-related factors MDA (C), SOD (D), and GSH (E) in ISO-induced H9c2 cells. (F-H) ELISA was performed to analyze the effect of lncRNA on the levels of inflammation-related factors IL-1 β , IL-6, and TNF- α in H9c2 cells induced by ISO. *, si-XIST *vs.* si-NC, $P < 0.05$; #, ov-XIST *vs.* ov-NC, $P < 0.05$. LDH, increased lactate dehydrogenase; ISO, isoproterenol; si, small interfering; NC, negative control; XIST, X-inactive specific transcript; ov, overexpression; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; IL, interleukin; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; lncRNA, long non-coding RNA.

oxidative stress, and promotion of inflammation. This effect was reversed by the ov of XIST (Figure 5A-5K).

Discussion

Globally, the mortality rate of patients with CHF is very

high (27,28), and its underlying pathological mechanisms remain unknown, which makes studying CHF crucial. Numerous studies have employed TCM or TCM extracts to effectively treat various diseases, such as chronic kidney disease (29) and colitis (30). Therefore, we used TCM as the starting point to analyze the role and potential

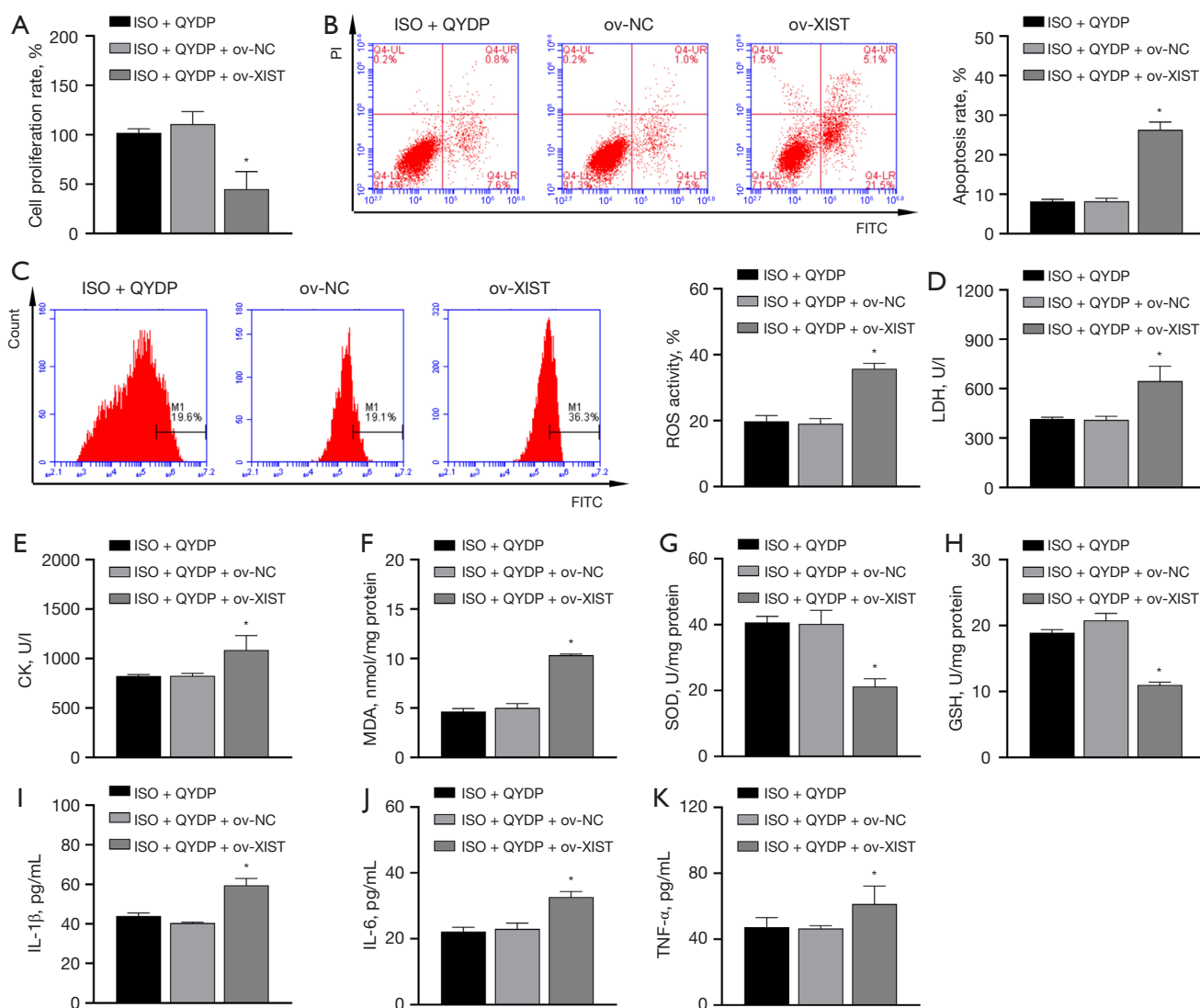


Figure 5 QYDP/lncRNA regulates the proliferation, apoptosis, oxidative stress, and inflammation of H9c2 cells induced by ISO. (A) A CCK8 assay was performed to assess the effect of QYDP/lncRNA on the proliferation rate of ISO-induced H9c2 cells. (B,C) FCM was performed to analyze the effect of QYDP/lncRNA on ISO-induced apoptosis (B) and ROS levels (C) of H9c2 cells. (D,E) ELISA was performed to analyze the effect of QYDP/lncRNA on the levels of myocardial enzyme-related factors LDH (D) and CK (E) in ISO-induced H9c2 cells. (F-H) ELISA was performed to analyze the effect of QYDP/lncRNA on the levels of oxidative stress-related factors MDA (F), SOD (G), and GSH (H) in ISO-induced H9c2 cells. (I-K) ELISA was performed to analyze the effect of QYDP/lncRNA on the levels of inflammation-related factors IL-1 β (I), IL-6 (J), and TNF- α (K) in ISO-induced H9c2 cells. * $P < 0.05$. ISO, isoproterenol; QYDP, Qishen Yiqi Dropping Pills; ov, overexpression; NC, negative control; XIST, X-inactive specific transcript; PI, propidium iodide; FITC, fluorescein isothiocyanate; ROS, reactive oxygen species; LDH, increased lactate dehydrogenase; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; IL, interleukin; TNF, tumor necrosis factor; lncRNA, long non-coding RNA; CCK8, cell counting kit-8; FCM, flow cytometry; ELISA, enzyme-linked immunosorbent assay.

mechanism of TCM in CHF. Wang *et al.* (31) used a variety of TCMs (such as Danshen, *P. notoginseng*, Angelica, and Astragalus) to screen for a more suitable treatment for

CHF. They found that *P. notoginseng* exerts anti-apoptotic effects, Danshen possesses anti-oxidative stress properties, and Astragalus exerts anti-inflammatory effects. The main

components of QYDPs are *P. notoginseng*, Danshen, and Astragalus; therefore, QYDPs are a reasonable choice for the treatment of CHF.

An important finding of this study was that ISO induced an increase in the expression of XIST, reduced the proliferation rate of H9c2 cells, and increased apoptosis and myocardial enzyme levels. These results are similar to those reported by Xiao *et al.* (32). In addition, QYDPs alleviated ISO-induced effects and partially restored the expression of XIST. These results suggest that XIST is closely related to the damage process in H9c2 cells. In addition, we found that QYDPs improved the oxidative stress and inflammation induced and promoted by ISO, which is consistent with the results of Cinar *et al.* (33). These results suggest that the role of QYDPs in protecting H9c2 cells involves oxidative stress and inflammation, but the mechanism of action of XIST remains unclear.

XIST may be involved in oxidative stress and inflammation to regulate cell proliferation and apoptosis (34,35). In this study, we investigated the effect of XIST on oxidative stress and inflammation, as well as its potential mechanism, by regulating the expression of XIST. In subsequent experiments, we observed that XIST could regulate the ISO-induced effects on cell proliferation, apoptosis, myocardial enzyme levels, oxidative stress, and inflammation in H9c2 cells. This provides new evidence that XIST induces cell damage by promoting oxidative stress and inflammation. Finally, we observed that the effect of QYDPs on the improvement of ISO induction in H9c2 cells was reversed by XIST. Thus, QYDP can improve the effect of ISO induction through XIST. Therefore, our study shows that QYDP may have a positive effect on the treatment and prognosis of CHF.

This study had several limitations that should be noted. Firstly, we did not analyze the effects of QYDPs on oxidative stress and inflammation in an animal model. Second, we did not conduct an in-depth analysis of the mechanism through which XIST acts as an endogenous competitive RNA to adsorb microRNAs to coregulate cell proliferation and apoptosis. Moreover, the process of lncRNA entering the clinic is hindered by issues related to specificity (off-target effects), delivery mode, and immunogenicity, which limit the clinical development of lncRNA therapeutics.

In summary, QYDPs protected H9c2 cells from damage caused by ISO-induced oxidative stress and inflammation by suppressing the expression of XIST. Therefore, this study confirmed that QYDP, as a new therapeutic drug for CHF,

has a solid pharmacological and molecular biological basis for the treatment of CHF.

Acknowledgments

Funding: This work was supported by the Hainan Provincial Key Research and Development Program (grant No. ZDYF2019121).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-606/rc>

Data Sharing Statement: Available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-606/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-22-606/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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(English Language Editor: A. Kassem)

Cite this article as: Luo Y, Chen J, Chen Y, Su Y, Wu X, Zheng W, Liu X, Chen L. Qishen Yiqi dropping pills improve isoproterenol-induced cardiomyocyte hypertrophy by regulating X-inactive specific transcript (XIST) expression in rats. *J Thorac Dis* 2022;14(6):2213-2223. doi: 10.21037/jtd-22-606