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*Original Article*

# Homocysteine induces cardiac hypertrophy by up-regulating ATP7a expression

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**Abstract:** Aims: The aim of the study is to investigate the molecular mechanism by which homocysteine (Hcy) induces cardiac hypertrophy. Methods: Primary cardiomyocytes were obtained from baby Sprague-Dawley rats within 3 days after birth. Flow cytometry was used to measure cell sizes. Quantitative real-time polymerase chain reaction was performed to measure the expression of β-myosin heavy chain and atrial natriuretic peptide genes. Western blotting assay was employed to determine ATP7a protein expression. Cytochrome C oxidase (COX) activity test was used to evaluate the activity of COX. Atomic absorption spectroscopy was performed to determine copper content. siRNAs were used to target-silence the expression of ATP7a. Results: Hcy induced cardiac hypertrophy and increased the expression of cardiac hypertrophy-related genes. ATP7a was a key factor in cardiac hypertrophy induced by Hcy. Reduced ATP7a expression inhibited cardiac hypertrophy induced by Hcy. Elevated ATP7a expression induced by Hcy inhibited COX activity. Enhanced ATP7a expression inhibited COX activity by lowering intracellular copper content. Conclusions: Hcy elevates ATP7a protein expression, reduces copper content, and lowers COX activity, finally leading to cardiac hypertrophy.

**Keywords:** Homocysteine, cardiac hypertrophy, ATP7a, cytochrome C oxidase, copper content

## Introduction

Homocysteine (Hcy) is an independent risk factor of cardiovascular diseases [1]. It is closely related to the occurrence of pathological lesions in cardiac muscle [2-4]. Clinical study shows that the content of Hcy in the blood of patients with cardiac hypertrophy is significantly elevated [5]. However, the molecular mechanism on how Hcy induces cardiac hypertrophy is still unclear.

Study shows that high concentration of Hcy leads to decreased activity of cytochrome C oxidase (COX) [6]. COX, also named mitochondrial respiratory membrane protein complex II, is a key enzyme on the mitochondrial respiratory chain that acts as the active center of mitochondrial functions [7-9]. COX receives four electrons from cytochrome C, passes the electrons to oxygen molecules, and transforms oxygen into two water molecules, finally producing adenosine triphosphate (ATP) that is necessary for all life events [10, 11]. Reduced COX activity leads to the dysfunction of mitochondria, which results in cardiac hypertrophy [12-15]. Therefore, decrease in COX activity might be the key mechanism of cardiac hypertrophy induced by Hcy.

The activity of COX is closely related to copper (Cu). Researchers show that reduced amount of Cu that is passed to COX can lead to significantly decreased COX activity [16-20]. Cu-transporting ATPase 1 (ATP7a) is an important protein that regulates intracellular Cu content, and maintains intracellular Cu delivery and homeostasis [21-23]. In the present study, we test the hypothesis that Hcy inhibits COX activity by regulating ATP7a expression, finally leading to cardiac hypertrophy.

## Materials and methods

### *Cells*

Cardiac apex parts were excised from baby Sprague-Dawley rats within 3 days after birth, cut into 1 $mm^3$ pieces and placed in penicillin. The tissues were rinsed twice with phosphate-buffered saline containing no calcium or magnesium ions, before being moved into 10 ml centrifuge tubes. Myocardial tissues were digested with trypsin and type II collagenase, and cardiomyocytes were collected by centrifugation at 200 ×g for 5 min. The cells were cultured in Hepes-buffered Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum at 37°C in the presence of 5% $CO_2$. For Hcy group, cells were cultured in serum-free medium containing 1 mM Hcy for 48 h, while cells in the control group were cultured in serum-free medium without Hcy for 48 h.

To test the effect of reduced ATP7a expression on cardiac hypertrophy induced by Hcy, cardiac muscle cells were cultured in Hepes-buffered Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum at 37°C in the presence of 5% $CO_2$ and transfected with 30 nM siRNAs that could target-silence ATP7a, using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). Mismatched siRNA was also used for transfection. The cells were divided into four groups: i) cells were cultured in serum-free medium for 96 h (control group); ii) cells were cultured in serum-free medium for 48 h, followed by addition of 1 mM Hcy and cultivation for another 48 h; iii) at 48 h after transfection with ATP7a siRNA, the cells were cultured in serum-free medium for another 48 h (ATP7a siRNA group); iv) at 48 h after transfection with ATP7a siRNA, the cells were cultured in serum-free medium containing 1 mM Hcy for another 48 h (Hcy + ATP7a siRNA group). The three siRNAs that target-silence ATP7a were: i) 5'-GAGGGCAUGUAGACGGUUA dTdT-3' and 3'-dTdT CUCCCGUACAUCUGCCAAU-5'; ii) 5'-ACAAUAUGCUACCUCCAAA dTdT-3' and 3'-dTdT UGUUAUACGAUGGAGGUUU-5'; and iii) 5'-GACUGGAUCCAUACAAUAU dTdT-3' and 3'-dTdT CUGACCUAGGUAUGUUAUA-5' (RiboBio, Guangzhou, China).

### *Flow cytometry*

After digestion with 1% TNE, the cells were suspended in Hepes-buffered Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum and stored in Eppendorf tubes for centrifugation at 200 ×g for 5 min. After discarding the supernatant, the cells were resuspended in phosphate-buffered saline containing no calcium or magnesium ions. Flow cytometry was performed according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA).

### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Platelet miRNA expression was determined by SYBR Green qRT-PCR (LightCycler® 480, Roche, Basel, Switzerland), using GAPDH as internal control. cDNA was synthesized from 1 μg total RNA using M-MLV reverse transcription kit (Takara, Dalian, China). The primer sequences for β-myosin heavy chain were 5'-TTGGCACGGACTGCGTCATC-3' (forward) and 5'-GAGCCTCCAGAGTTTGCTGAAGGA-3' (reverse). The primer sequences for atrial natriuretic peptide were 5'-ATCTGATGGATTTCAAGAACC-3' (forward) and 5'-CTCTGAGACGGGTTGACTTC-3' (reverse). The primer sequences for GAPDH were 5'-CATGTACGTTGCTATCCAGGC-3' (forward) and 5'-CTCCTTAATGTCACGCACGAT-3' (reverse). The reaction system contains 12.5 μl SYBR Premix Ex Taq (Takara, Dalian, China), 1 μl PCR Forward Primer, 1 μl Uni-miR qPCR Primer, 2 μl templates, and 8.5 μl double-distilled $H_2O$ (a total of 25 μl). Each sample was tested in triplicate. The PCR protocol was as follows: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of miRNA.

### *Western blotting*

Protein samples (30 μg) were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (300 mA, 1.5 h) and blocked with 50 g/L skimmed milk at room temperature for 1 h. Then, the membranes were incubated with ATP7a antibody (1:1000) and GAPDH antibody (1:2000) (Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit-horseradish peroxidase conjugate antibody (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at

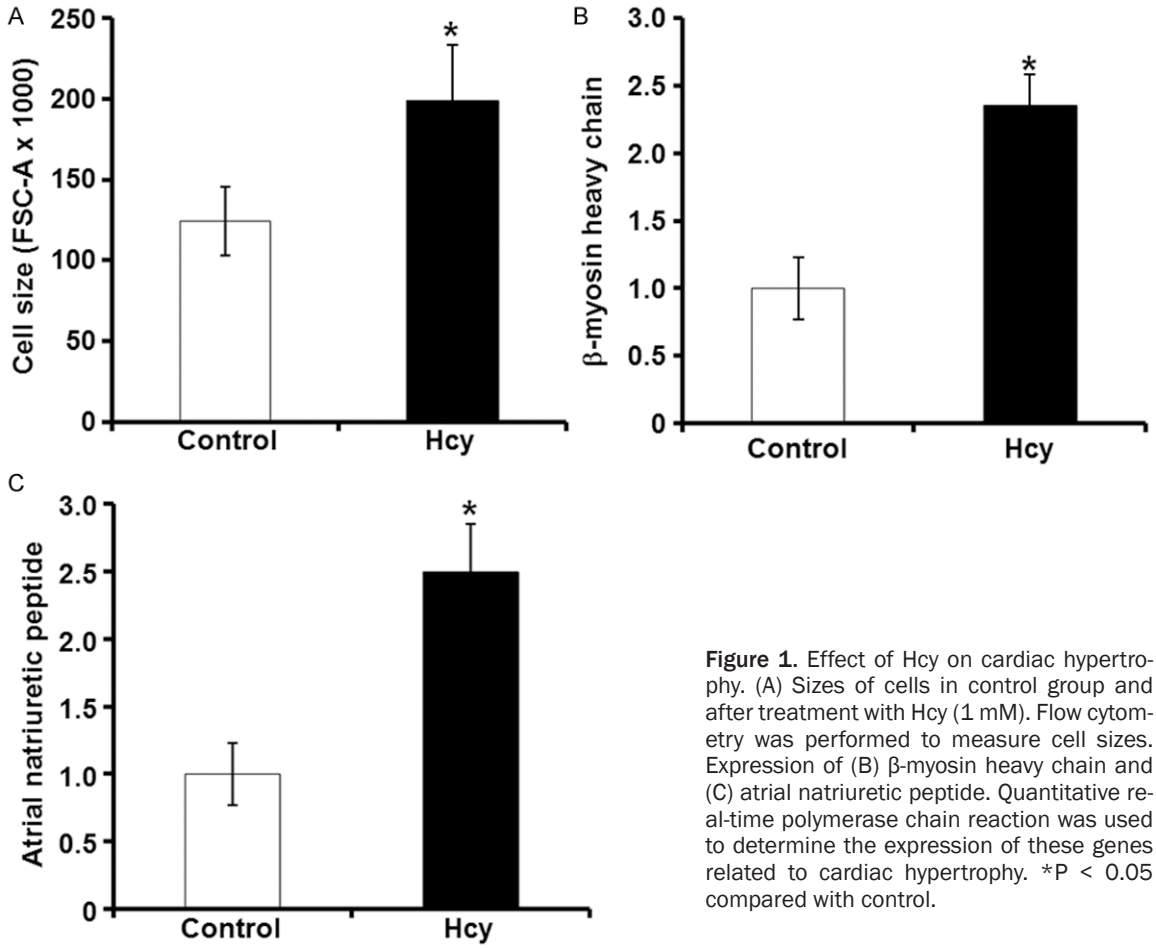


**Figure 1.** Effect of Hcy on cardiac hypertrophy. (A) Sizes of cells in control group and after treatment with Hcy (1 mM). Flow cytometry was performed to measure cell sizes. Expression of (B) β-myosin heavy chain and (C) atrial natriuretic peptide. Quantitative real-time polymerase chain reaction was used to determine the expression of these genes related to cardiac hypertrophy. *P < 0.05 compared with control.

room temperature before washing with phosphate-buffered saline with Tween 20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab (Bio-Rad, Hercules, CA, USA) software was used to acquire and analyze imaging signals. The relative content of ATP7a protein was expressed as ATP7a/GAPDH ratio.

*COX activity test*

Cardiomyocytes were trypsinized and collected by centrifugation (200 ×g) in Eppendorf tubes. The cells were then resuspended in 1 ml mitochondrial separation medium buffer, and transferred to Dounce homogenizer (PRO Scientific, Oxford, CT, USA) for homogenization on ice for 200 times. Cell homogenates were then transferred to a new 2 ml Eppendorf tube for centrifugation at 600 ×g for 5 min. The supernatant was then centrifuged at 4°C and 1000 ×g for 5 min. Subsequently, the supernatant was collected for another centrifugation at 4°C and 7000 ×g for 10 min. After discarding supernatant, the sediments were then resuspended in 1 ml mitochondrial separation medium buffer, followed by centrifugation at 4°C and 7000 ×g for 10 min. Then the supernatant was discarded and the sediments were resuspended in 1 ml MSTE buffer, followed by centrifugation at 4°C and 10000 ×g for 10 min to collect mitochondria.

The collected mitochondria samples were mixed with 40 μl 1% sodium dodecyl sulfate and place on ice. The mixture was then vortexed for 10 s every 5 min, for a total of 6 times and 30 min. Then, the mixture was centrifuged at 4°C and 12000 ×g for 10 min to collect supernatant, which was mitochondrial protein lysates. The concentration of mitochondrial protein was determined using DAC protein detection kit (Bio-Rad, Hercules, CA, USA). COX activity was tested using COX activity detection kit (Genmed, Shanghai, China).

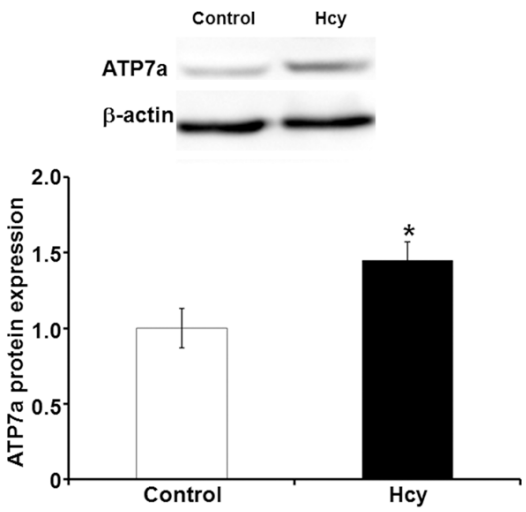


**Figure 2.** Effect of Hcy on ATP7a protein expression. ATP7a protein expression in cells of control group and after treatment with Hcy (1 mM) was determined by Western blotting. β-actin was used as internal reference. ATP7a level in Hcy group was normalized to that in control. *P < 0.05 compared with control.

*Atomic absorption spectroscopy*

After digestion with 1% TNE, cardiomyocytes were suspended in 100 μl 1% sodium dodecyl sulfate followed by vortexing. The cells were lysed by sonication and centrifuged at 4°C and 12000 ×g for 10 min. The concentrations of proteins in the supernatant were determined using DAC protein detection kit (Bio-Rad, Hercules, CA, USA). The extracted protein solution was mixed with concentrated nitric acid (v:v, 1:5) and left standing for 2 days. The content of Cu was determined by atomic absorption spectroscopy, followed by normalization to the total protein content in cardiomyocytes.

*Statistical analysis*

All data were analyzed using SPSS 13.0 statistical software (IBM, Chicago, USA). Differences between groups of data were compared using t-test. $P < 0.05$ was considered statistically significant.

## Results

*Hcy induces cardiac hypertrophy and increases the expression of cardiac hypertrophy-related genes*

To test the effect of Hcy on cardiac hypertrophy, flow cytometry was used to determine cell sizes, and qRT-PCR was performed to measure the expression of β-MHC and ANP genes that are related to cardiac hypertrophy. Flow cytometry data showed that Hcy (1 mM) induced significantly enlarged cardiomyocyte sizes ($P < 0.05$) (**Figure 1A**). qRT-PCR data showed that the expression of both β-MHC and ANP was significantly increased by 1 mM Hcy ($P < 0.05$) (**Figure 1B** and **1C**). These results suggest that Hcy induces cardiac hypertrophy and increases the expression of cardiac hypertrophy-related genes.

*ATP7a is a key factor in cardiac hypertrophy induced by Hcy*

To determine ATP7a protein expression in cardiomyocytes, Western blotting assay was performed. The data showed that ATP7a protein levels in cardiomyocytes incubated with 1 mM Hcy were significantly higher than control ($P < 0.05$) (**Figure 2**). These results indicate that ATP7a is a key factor in cardiac hypertrophy induced by Hcy.

*Reduced ATP7a expression inhibits cardiac hypertrophy induced by Hcy*

To measure cell sizes and expression of ATP7a, β-MHC and ANP, flow cytometry and qRT-PCR were performed, respectively. The data showed that ATP7a expression in mismatched siRNA group was not different from control, while cells transfected with ATP7a siRNA had significantly reduced ATP7a expression compared with control ($P < 0.05$) (**Figure 3A**). After treatment with 1 mM Hcy, cell sizes and expression of β-MHC and ANP were significantly enhanced compared with control. Furthermore, cell sizes and expression of β-MHC and ANP in the ATP7a siRNA group were not different from control, and treatment with 1 mM Hcy significantly increased cell sizes and expression of β-MHC and ANP compared with ATP7a siRNA group ($P < 0.05$). Of note, the effect of 1 mM Hcy on ATP7a siRNA group was partially inhibited compared with its effect on control group ($P < 0.05$) (**Figure 3B-D**). These results suggest that reduced ATP7a expression inhibits cardiac hypertrophy induced by Hcy.

*Elevated ATP7a expression induced by Hcy inhibits COX activity*

To test whether ATP7a affects COX activity, COX activity test was performed. The data showed

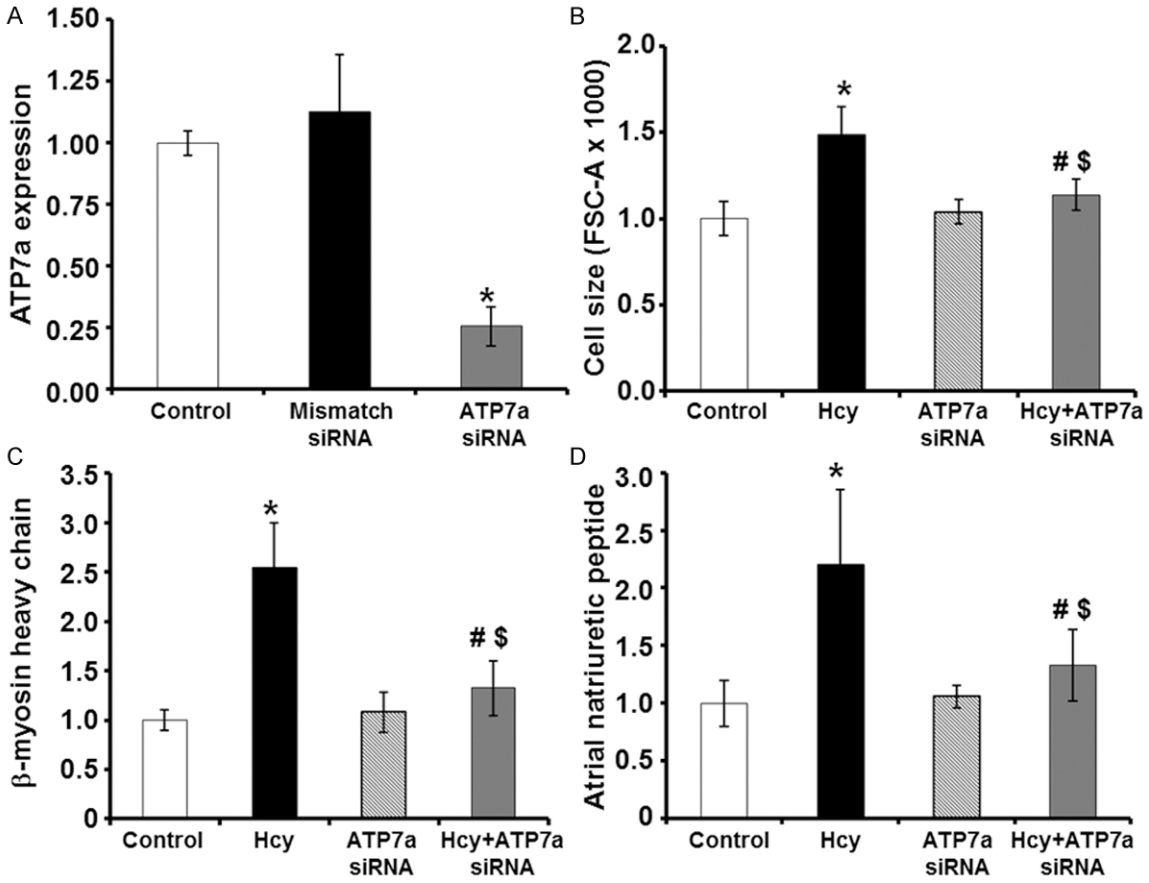


**Figure 3.** Effect of ATP7a silencing on cardiac hypertrophy induced by Hcy. (A) Expression of ATP7a in control, mismatched siRNA and ATP7a siRNA groups. Quantitative real-time polymerase chain reaction was used to determine the expression of ATP7a. (B) Sizes of cells in control, Hcy, ATP7a siRNA and Hcy + ATP7a siRNA groups. Flow cytometry was performed to measure cell sizes. Expression of (C) β-myosin heavy chain and (D) atrial natriuretic peptide in control, Hcy, ATP7a siRNA and Hcy + ATP7a siRNA groups. Quantitative real-time polymerase chain reaction was used to determine the expression of these genes. Hcy concentration was 1 mM wherever used in the experiments. *P < 0.05 compared with control; #P < 0.05 compared with ATP7a siRNA group; $P < 0.05 compared with Hcy group.

that 1 mM Hcy significantly reduced COX activity ($P < 0.05$), while transfection with ATP7a siRNA had no significant effect on COX activity ($P > 0.05$). Treatment with 1 mM Hcy also significantly reduced COX activity in cells transfected with ATP7a siRNA ($P < 0.05$), but had smaller effect compared with Hcy treatment on cells without ATP7a siRNA transfection. COX activity in Hcy + ATP7a siRNA was significantly higher than that in Hcy group ($P < 0.05$) (**Figure 4A**). These results indicate that elevated ATP7a expression induced by Hcy inhibits COX activity, leading to cardiac hypertrophy.

*Enhanced ATP7a expression inhibits COX activity by lowering intracellular Cu content*

To measure whether ATP7a expression is related to Cu content, we performed atomic absorption spectroscopy. The data showed that 1 mM Hcy significantly reduced Cu content compared with control ($P < 0.05$). In addition, Cu content in cells transfected with ATP7a siRNA was not significantly different from that in control ($P > 0.05$). Cu content in Hcy + ATP7a siRNA group was lower than that in ATP7a siRNA group ($P < 0.05$), but 1 mM Hcy had smaller effect on ATP7a siRNA group compared with control group. Cu content in Hcy + ATP7a siRNA was significantly higher than that in Hcy group ($P < 0.05$) (**Figure 4B**). These results suggest that enhanced ATP7a expression inhibits COX activity by lowering intracellular Cu content.

## Discussion

Hcy plays important roles in the occurrence and development of cardiovascular disease

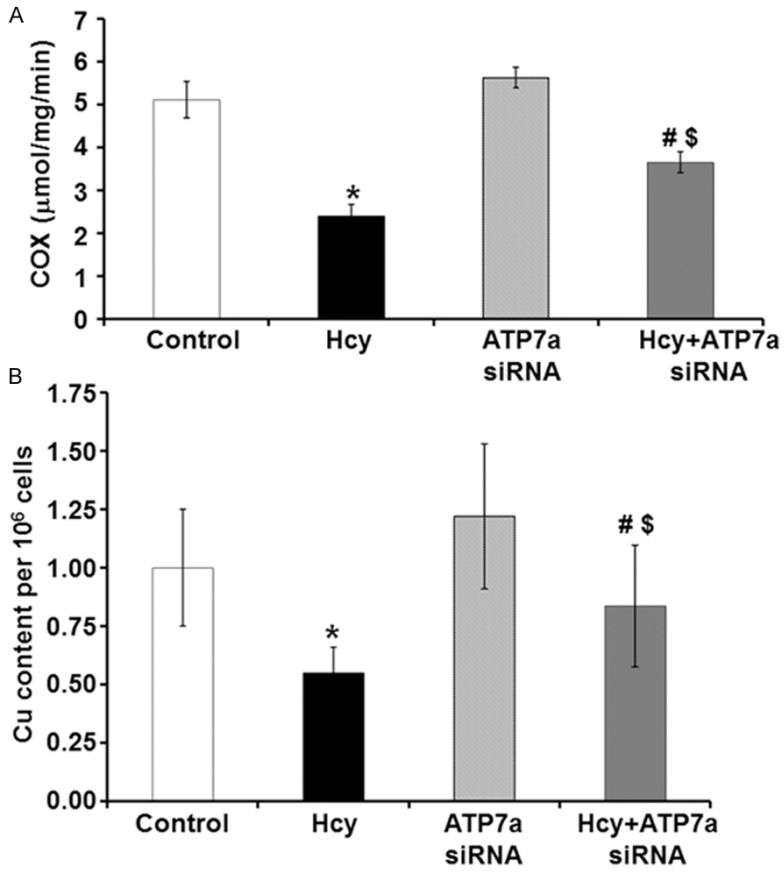


**Figure 4.** Effect of ATP7a silencing on (A) cytochrome C oxidase activity and (B) intracellular copper content. Cytochrome C oxidase acitivty and intracellular copper content in control, Hcy, ATP7a siRNA and Hcy + ATP7a siRNA groups were measured using cytochrome C oxidase activity test and atomic absorption spectroscopy, respectively. Hcy concentration was 1 mM wherever used in the experiments. *P < 0.05 compared with control; #P < 0.05 compared with ATP7a siRNA group; $P < 0.05 compared with Hcy group.

[1-5]. The present study found that Hcy induced significantly enlarged cardiomyocyte sizes, as well as significantly enhanced expression of cardiac hypertrophy-related genes β-MHC and ANP. In addition, Western blotting analysis showed increased ATP7a protein expression. When siRNAs that target ATP7a were used to silence ATP7a, cardiac hypertrophy induced by Hcy was significantly inhibited, suggesting that ATP7a plays important roles in this process.

COX is an important factor that leads to cardiac hypertrophy and its activity is dependent on Cu [16-20]. After Cu enters the cells via Ctr1, three metabolic pathways follow. First, Cu is passed to CuZn-superoxide dismutase via copper chaperone and participates in oxidative stress reactions. Second, Cu is passed to COX via COX17 and participates in energy metabolism. Third, Cu is passed to ATP7a via antioxidant protein 1 and participates in protein synthesis and secretion. Our study shows that increased ATP7a not only reduces Cu content, but also decreases COX activity. It is possible that ATP7a occupies the Cu that should have been passed to COX. However, the present study did not elucidate the mechanism by which Hcy increases ATP7a expression. This will be our future research concerns. To summarize, ATP7a plays important roles in cardiac hypertrophy induced by Hcy. Hcy induces ATP7a protein expression, decreases intracellular Cu content, decreases COX activity, and finally leads to cardiac hypertrophy. Inhibition of ATP7a expression suppresses the effect of Hcy in decreasing COX activity and Cu content, and thus inhibits the effect of Hcy in inducing cardiac hypertrophy.

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## Disclosure of conflict of interest

None.

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