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Effect of electroacupuncture "Shenmen"HT7 on the expression of ferroptosis related proteins GPX4, FTH1, TfR1 and ACSL4 in Acute Myocardial Ischemia Model Rats

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Abstract

Objective: To observe the effect of electroacupuncture at the "Shenmen"(HT7) acupoint of the Heart Meridian on the related proteins of "ferroptosis" in the myocardium of (AMI) rats with myocardial ischemia.

Methods: Thirty-six healthy adult SD rats, weighing (230g ±20g), were randomly divided into three groups: sham operation group (Sham), model group (Model) and electroacupuncture group (EA), to establish acute myocardial ischemia rat (AMI). The rat model of acute myocardial ischemia was established by ligating the left anterior descending branch of coronary artery. The rats in the sham operation group were fed normally for 7 days after modeling, and the rats in the electroacupuncture group were stimulated by electroacupuncture for 7 days with a stimulation current of 1mA and a frequency of 2 Hz for 30min. In the sham operation group, the rats in the model group were fed normally for 7 days after modeling. The electrocardiogram of rats in each group was recorded and analyzed by PowerLab 16-lead physiological recorder. 7 days later, 6 rats in each group were taken from the left ventricle and killed. The ultrastructure of the apical tissue was observed under transmission electron microscope, the pathological changes of myocardial tissue were observed by HE staining, and the activities of Fe^{2+} and glutathione (GSH) in myocardial tissue were determined by colorimetry. The mRNA expression levels of glutathione peroxidase 4 (GPX4), ferritin heavy chain polypeptide 1 (FTH1), transferrin receptor 1 (TfR1) and long chain acyl-CoA synthase 4 (ACSL4) in rat myocardium were detected by real-time fluorescence quantitative PCR (QT-PCR). The expressions of GPX4, FTH1, TFR1 and ACSL4 in rat myocardium were detected by Western blot.

Results: Compared with the sham operation group, the electrocardiogram ST segment of the model group was obviously elevated, the arrangement of cells in myocardial tissue was disordered, the myocardial fiber was broken, the interstitial hemorrhage was obvious, the mitochondrial atrophy became smaller, the membrane density increased, the myocardial Fe^{2+} content increased and the GSH activity decreased, and the expression levels of GPX4, FTH1 mRNA and protein in myocardial tissue decreased ($P < 0.01$). The expression level of TFR1 and ACSL4 and the expression of protein were increased ($P < 0.01$). Compared with the model group, the ST segment decreased significantly, the arrangement of cardiomyocytes, the breakage of myocardial fibers, the interstitial hemorrhage decreased ($P < 0.01$), the mitochondrial atrophy decreased, the membrane density increased, the content of Fe^{2+} decreased and the activity of GSH increased in the electroacupuncture group. The expression level of GPX4, FTH1 mRNA and protein in myocardial tissue increased ($P < 0.01$), while the expression level of TFR1 and ACSL4 and the expression of protein decreased in the electroacupuncture group.

Conclusion: The protective effect of acupuncture on the myocardium of AMI rats may be related to the inhibition of "ferroptosis" by affecting the expression of

myocardial related proteins GPX4, FTH1, TfR1 and ACSL4.

Keywords： Electroacupuncture; HT7; AMI; Mechanism ; Ferroptosis

Introduction

Acute myocardial ischemia ((AMI)) is a common cardiovascular disease in clinic, which is often characterized by decreased blood supply and oxygen supply and abnormal energy metabolism (Lindsey et al., 2018). In recent years, due to the high morbidity and mortality, it has become a worldwide epidemic (Zhao et al., 2019; Weintraub et al., 2019). Preventing and reducing cardiomyocyte death is the key to improve and restore cardiac function. Acupuncture has a unique effect of improving myocardial ischemia (Painovichy et al., 2014; Mehta et al., 2014). A large number of experimental studies have shown that acupuncture has a clear cardioprotective effect, and many scholars at home and abroad have also improved microcirculation, anti-oxygen free radicals, regulation of cytokines, and effects on NOS and NO. And regulating the expression of heat shock protein genes to explore the effect and mechanism of acupuncture on myocardial ischemia (Fan et al., 2015). "Ferroptosis" is a previously unknown regulatory mechanism and signal transduction pathway of regulatory cell death discovered in recent years. 2019 revealed the role of "ferroptosis" in heart injury and its molecular mechanism of heart disease. It is proved that not only iron death exists in heart injury (Fang et al., 2019), but also targeted intervention of iron death can effectively prevent the occurrence and development of heart disease. "Ferroptosis" is a new mode of cell death, which is different from necrosis, apoptosis, autophagy, pyrogenesis and so on. It is a mode of death mainly caused by mitochondrial changes caused by excessive accumulation of iron-dependent lipid peroxides (Dixon et al., 2012). Iron homeostasis regulation pathway is activated after myocardial ischemia, iron ion accumulation and active oxygen production eventually lead to iron-dependent oxidative damage, that is ferroptosis. GPX4, FTH1, TfR1, ACSL4 and other related proteins are involved in the process of myocardial ischemic injury in rats. In this study, the role of "ferroptosis" related proteins in electroacupuncture in reducing myocardial injury in rats, to further clarify the mechanism of acupuncture to protect the heart.

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1 Materials and methods

1.1 Animals

Thirty-six clean-grade healthy male SD rats, weighing (230g ±20g), were provided by the feeding Center of Anhui Medical University, license No.: SCXK (Wan) 2019-003. Adaptive feeding for 1-week, indoor temperature (21 ±1) ℃, humidity 40%-60%, free to eat and drink. The rats were randomly divided into sham operation group (n = 12), model group (n = 12) and electroacupuncture group (n = 12). The experimental process strictly followed the guiding opinions on being kind to Experimental Animals issued by the Ministry of Science and Technology of the people's Republic of China in 2006, and the whole experimental process was reviewed by the Ethical Review Committee of Experimental Animals of Anhui University of traditional Chinese Medicine. make every effort to minimize the number and suffering of rats.

1.2 Reagents and instruments

Isoflurane (Shenzhen Rivard Life Technology Co., Ltd); 0.9% sodium chloride solution (Anhui Shuanghe Pharmaceutical Co., Ltd.); BCA protein concentration determination kit (enhanced) (batch number: 09191919191205, Beyotime); microreduced glutathione (GSH) (batch number: 20200731, Nanjing Jiancheng Biological Engineering Research Institute). Tissue iron (Fe) determination kit (batch number: 20200706, Nanjing Jiancheng Biological Engineering Research Institute) Western primary and secondary antibody removal solution (batch number: 051418180626, Shanghai Biyuntian); β-Actin (batch number: 051418180626 Zsbio); GPX4 antibody (batch number: AH082217,Bioss); FTH1 antibody (batch number: GR217524-6, British Abcam); TFR1 antibody (batch number: AI071124,Bioss); ACSL4 antibody (batch number: H2119 Santa Cruz); HT7800 transmission electron microscope (Hita-chi, Japan). Fluorescent quantitative PCR instrument (model: PIKOREAL96, Thermo Scientific); slicer (Thermoscientific company); high-speed desktop freezing centrifuge (model: JW-3021HR, Anhui Jiawen instrument and equipment Co, Ltd); electrothermal incubator (model: DNP-9052BS- III, Shanghai Sanfa); disposable acupuncture needle (0.25 mm × 13 mm, Suzhou Medical supplies Co.,

Ltd); Huachi brand SDZ electronic acupuncture instrument (Suzhou Medical supplies Co., Ltd).

2 Methods

2.1 Preparation of animal model

The rats were anesthetized with isoflurane gas, and the induction concentration was adjusted to 3%-4%. 1 min, after the anesthetic was filled with the induction box, the rats were put into the induction box for complete anesthesia, and their heads and noses were placed on the anesthetic mask to maintain a concentration of 2%-2.5%. In the supine position, the skin of the chest skin was prepared, the deep pectoralis muscle and superficial pectoralis muscle were bluntly separated, the 4th and 5th intercostal muscles were opened with hemostatic forceps, and the heart was exposed. The left anterior descending branch of coronary artery was identified and ligated with 6-0 suture needle. The apical tissue of rats became white, sutured and sprayed with penicillin sodium. Pseudo-operation group: the mode of anesthesia was the same as that of the model group, exposing the heart, only thread without ligation.

2.2 Intervention methods

With reference to the method of locating acupoints in human body and in combination with Experimental Acupuncture and moxibustion (Yu and Guo, 2014), the rat supine position is fixed on the mouse plate in the acupuncture and moxibustion acupoint positioning method commonly used in experimental animals. In the electroacupuncture group, bilateral "Shenmen acupoint" (HT7) was selected, the depth was 1mm to 2mm, and the electroacupuncture instrument was connected with electroacupuncture stimulation. The current intensity was 1 mA and the frequency were 10 Hz, once a day for 15 minutes each time. Electroacupuncture was started on the second day after the replication of the model for a total of 7 days. The sham operation group and the model group did not receive electroacupuncture.

2.3 Electrocardiogram examination

The second lead electrocardiogram of the limb was recorded by PowerLab Systems, and the electrode was punctured under the skin of the left posterior, right anterior and right hindlimb of rats. The ST segment of ECG was observed and recorded to judge whether the AMI model was successful or not.

2.4 Collection and preservation of specimens

After the last treatment, the heart was taken out after 1% pentobarbital sodium intraperitoneal anesthesia, and part of the fresh tissue was fixed in 4% paraformaldehyde for 24 hours. the apical part of the heart was cut into 1mm × 1mm × 1mm and fixed with 2.5% glutaraldehyde for 24 hours. 0.9% sodium chloride washed the remaining blood and put it into the cryopreservation tube and stored at-80 ℃ for PCR and Western Blot detection of myocardial tissue Fe^{2+}, glutathione (GSH), GPX4, FTH1, TfR1, ACSL4.

2.5 Observation of myocardial histomorphology in rats by HE

After the rats were killed, the hearts of each group were fixed with conventional 4% polyoxymethylene for 48 hours, washed with distilled water for 10 minutes, dehydrated with gradient ethanol for 1.5 hours, immersed in xylene for 30 minutes each time, paraffin-embedded, sliced (thickness 4 um) and baked at 65 ℃. The slides were dewaxed, watered, stained, re-stained, transparent, sealed and photographed under light microscope to observe the routine pathological changes of myocardium.

2.6 Observation of mitochondrial morphology under transmission electron microscope.

After the last treatment, the apical myocardial tissue of each group was cut into about the size of 1mm × 1mm × 1mm, and the 1min was fixed with 2.5% glutaraldehyde for 24 hours. after being fixed with 1% osmic acid for 1.5 hours, the myocardial ultrastructure was observed under HT7800 transmission electron microscope.

2.7 Colorimetric determination of Fe^{2+} and Glutathione (GSH) activity in Rat Myocardium.

After the last treatment, 6 rats in each group were selected, and the apical myocardial tissue was made into tissue homogenate according to the instructions of BCA protein quantitative kit. The protein concentration was determined, and the activities of Fe2+ and glutathione (GSH) in rat myocardial tissue were detected.

2.8 The expression of GPX4, FTH1, TFR1 and ACSL4 mRNA in rat myocardium was detected by PCR method.

After the last treatment, 6 rats in each group were selected. The myocardial tissue was shredded and grinded with liquid nitrogen, and 1 mL Trizol homogenate was added. RNA was extracted and reverse transcribed and amplified by cDNA. The conditions were pre-denatured at 95 ℃ for 1 min, denatured at 95 ℃, denatured at 20 ℃, annealed

and extended at 60 ℃ for 1 min, 40 cycles. The sequences of upstream and downstream primers are shown in Table 1. Using β-actin gene as internal reference gene, the relative mRNA expression of GPX4, FTH1, TfR1 and ACSL4 was analyzed by 2-Ct method.

2.9 The protein contents of GPX4, FTH1, TfR1 and ACSL4 in myocardial tissue of rats were detected by Western Blot method

After the last treatment, 6 rats in each group were added with RIPA cell lysate for 1 mL to completely lyse the tissue, and the supernatant was centrifuged for 10 min at 12000 r/min to extract total protein. Follow the instructions of BCA protein quantitative kit to determine the protein concentration. Add the sample buffer and boil at 100C for 10min, electrophoresis with polyacrylamide gel, wet transfer on PVDF film, 5% skimmed milk powder sealed at room temperature for 2h, add diluted GPX4, FTH1, TfR1, ACSL4 first antibody solution, incubate overnight at 4 ℃. PBST was washed three times for 10 minutes each, and the second antibody labeled by horseradish peroxidase (HRP) was diluted with the second antibody diluent. 10min was washed by PBST for 1.2 hours at room temperature for 3 times and each time. ECL luminescence kit was used to detect proteins, and Image J software was used to analyze the gray values of protein bands in each group.

Table 1. **Primers for each detection index**

Gene	Amplicon Size （bp）	Forward primer （5'→3'）	Reverse primer （5'→3'）
β-actin	150	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
ACSL4	93	GGCTTGAGATTCACAGCATG	TGTCCGAAGGAGTTGGTCTA
FTH1	179	CCAGAACTACCACCAGGACTC	GTTTCTCAGCATGTTCCCTCT
TFR1	139	ACTCTGCTTTGCGACTATTGC	TTCTGACTTGTCCGCCTCTT
GPX4	172	AATCCTGGCCTTCCCTTGCA	GCCCTTGGGCTGGACTTTCA

3 Results

3.1 The effect of electroacupuncture on electrocardiogram

Compared with the sham operation group, the ST segment increased significantly after 30 min ligation in the model group and acupuncture group, and the ST segment decreased significantly in the electroacupuncture group compared with the model group. See figure 1.

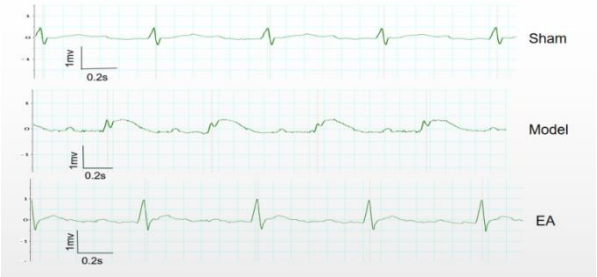

Figure 1**. Observation of electrocardiographic changes in rats of each group**

3.2 Pathological changes of myocardial tissue in HE rats

Compared with the pseudo-operation group, the arrangement of myocardial cells in the model group was disordered, the myocardial fiber was broken, and the interstitial hemorrhage was obvious. compared with the model group, the arrangement of cardiomyocytes and the rupture of myocardial fiber in the electroacupuncture group were improved, and the interstitial hemorrhage was reduced. See figure 2

3.3 Observation of mitochondrial morphology under transmission electron microscope

Compared with the sham operation group, the myocardial mitochondria atrophy decreased and the membrane density increased in the model group under transmission electron microscope. Compared with the model group, the myocardial mitochondria atrophy decreased and the membrane density increased in the electroacupuncture group under transmission electron microscope. See figure 3

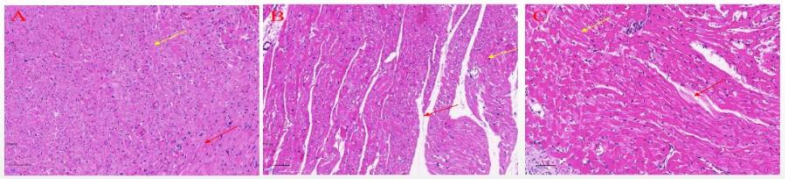

Figure 2. **Pathological observation of myocardial tissue of rats in each group (HE, scale = 50μm)**
Note: A, B, C show pseudo-operation group, model group and electroacupuncture group; yellow arrow shows cardiomyocyte, red arrow indicates myocardial fiber

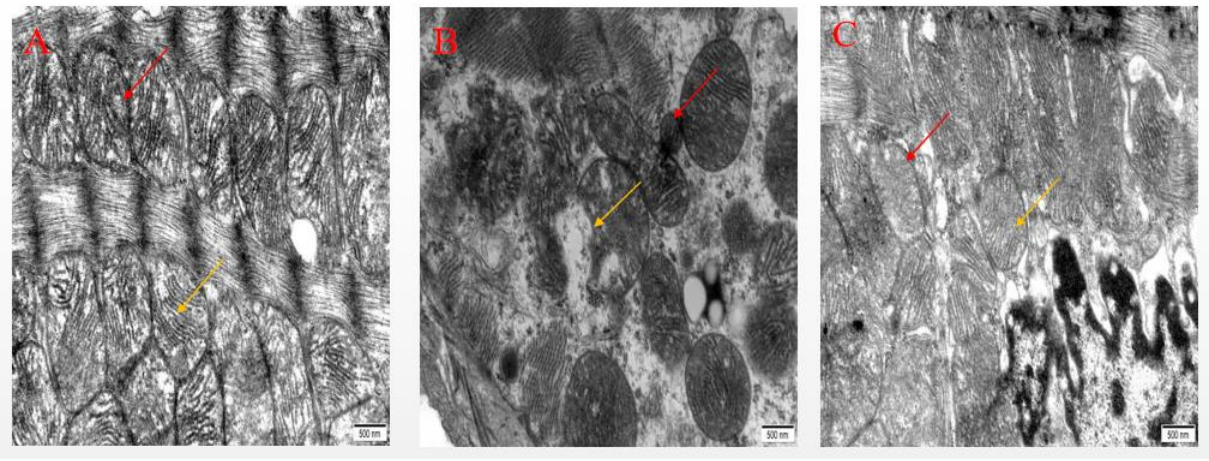

Figure 3. **Morphological observation of mitochondria of rats in each group (scale = 500nm)**
Note: A, B, C show pseudo-operation group, model group and electroacupuncture group; yellow arrow shows cardiomyocyte mitochondria, red arrow shows cardiomyocyte mitochondrial membrane structure

3.4 Determination of iron content and glutathione (GSH) activity in rat myocardial tissue by colorimetry

Compared with the sham operation group, the iron content in the myocardial tissue of the model group increased significantly and the activity of GSH decreased（*P*<0.01）, while the iron content of the myocardial tissue decreased and the activity of GSH increased in the electroacupuncture group compared with the model group（*P*<0.01）. See figure 4

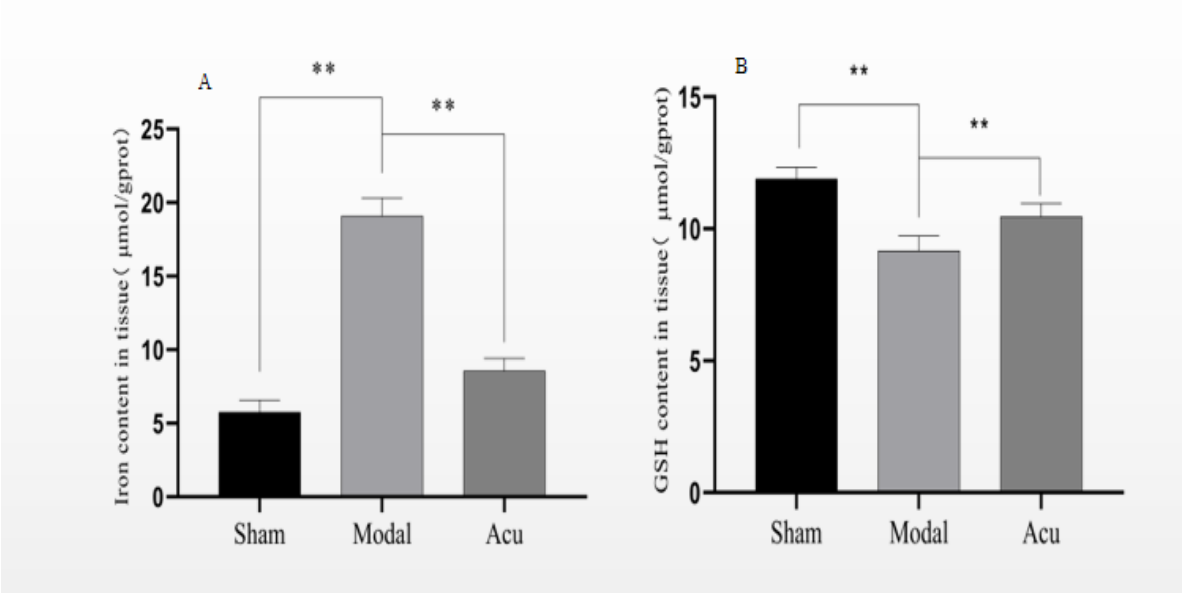

Figure4. **Iron content and glutathione (GSH) activity in myocardium of rats in each group**
Note: figure 4A shows iron content in myocardial tissue and figure 4B shows glutathione (GSH) activity***P*<0.01.

3.5 Effect of electroacupuncture on the expression of GPX4, FTH1, TfR1 and ACSL4 mRNA in Myocardium

Compared with the sham operation group, the expression level of GPX4 and FTH1mRNA in the model group decreased significantly（*P*<0.01）, while the expression level of TfR1 and ACSL4 mRNA increased significantly in the model group（*P*<0.01）, while the expression level of GPX4 and FTH1 mRNA increased significantly and the expression level of TfR1 and ACSL4 mRNA decreased significantly in the electroacupuncture group compared with the model group（*P*<0.01）. See figure 5

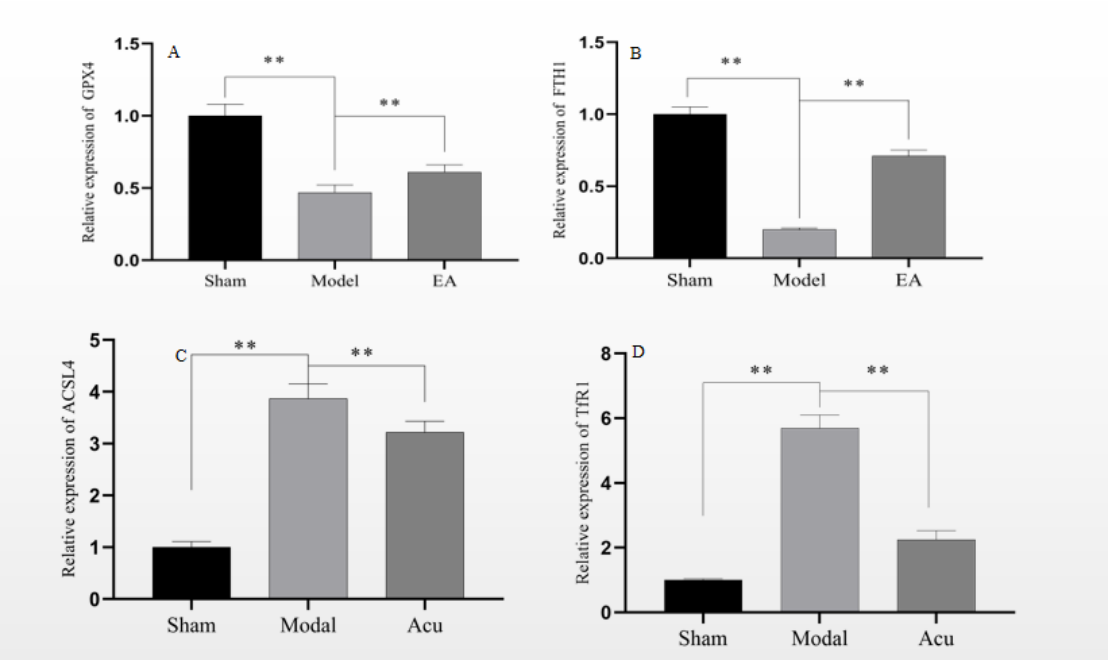

Figure 5. **Expression level of GPX4, FTH1, TfR1 and ACSL4 mRNA in myocardial tissue of rats in each group**
Note: figs. 5 A, B, C and D show the expression levels of GPX4, FTH1, TFR1 and ACSL4 mRNA in myocardial tissue**P<0.01.

3.6. Effect of electroacupuncture on protein expression of GPX4, FTH1, TfR1 and ACSL4 in Myocardium

Compared with the sham operation group, the protein expression levels of GPX4 and FTH1 in the model group were significantly decreased（P<0.01）, while the protein expression levels of TfR1 and ACSL4 were significantly increased in the model group（P<0.01）. Compared with the model group, the protein expression levels of GPX4 and FTH1 in the electroacupuncture group were significantly higher than those in the model group,（P<0.01） while the protein expression levels of TfR1 and ACSL4 in the electroacupuncture group were significantly lower than those in the model group（P<0.01）.

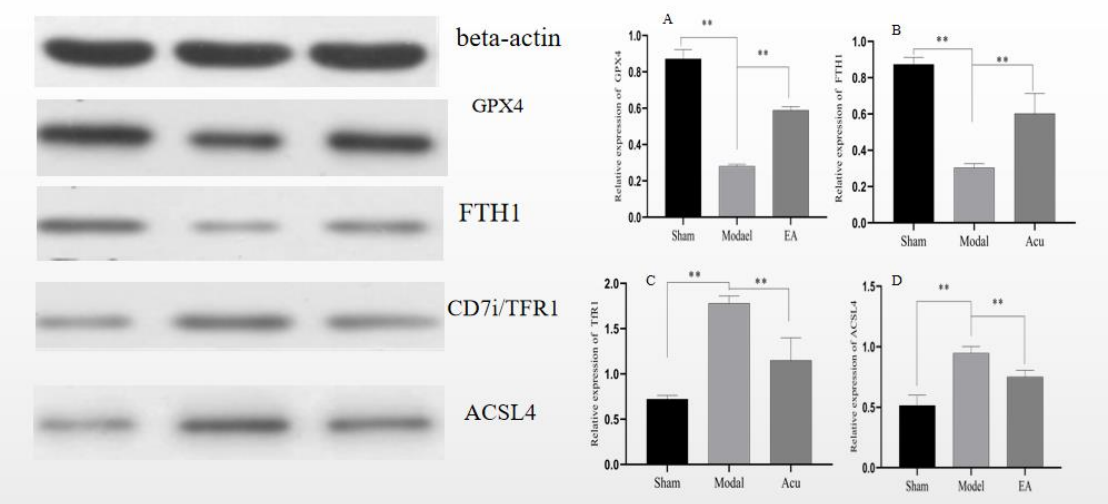

Figure 6. **Protein expression level of GPX4, FTH1, TfR1 and ACSL4 in myocardial tissue of rats in each group**
Note: Fig. 5 A, B, C and D show the protein expression levels of GPX4, FTH1, TfR1 and ACSL4 in myocardial tissue, respectively**P<0.01.

4 Discussion

As one of the traditional Chinese medical methods, the meridian is a complex signal transduction network system. Acupuncture can regulate the release of some chemicals or hormones through the changes of substance, energy and information flow of meridians. Acupuncture of the Heart Meridian can specifically improve cardiac function through nerves, blood vessels, body fluids and other ways. It has the characteristics of multi-layers and multi-targets (Kaptchuk, 2002) ;(Cheng, 2014) ;(Ni and Frishman, 2018). Shenmen (HT7) is a commonly used acupoint of Shao-Yin Heart meridian. It is often used to treat cardiovascular diseases. Clinical evidence shows that (Wu et al., 2018; Wang et al., 2018; Li and Liu, 2017) has a unique effect on angina pectoris, atrial fibrillation, cardiac neurosis, insomnia, hypertension and other cardiovascular and nervous system diseases. Previous experimental mechanism studies have confirmed that (Cai et al., 2007; Hao et al., 2020; Shi et al., 2019) acupuncture with Shenmen (HT7) can effectively improve cardiac function and cardiac sympathetic electrical activity in patients with myocardial infarction, reduce the content of serum myocardial enzymes, increase the level of gap protein in cardiomyocytes, inhibit apoptosis and improve damaged myocardium through antioxidant stress reaction, autophagy and inflammatory reaction. However, most of the research results exist in isolation, and the human body is an organic whole, and all parts coordinate with each other to maintain the normality of the body. Studies have shown that the occurrence of (Li et al., 2020) "ferroptosis" is closely related to autophagy, oxidative stress and inflammation, and studies have shown that there is not only "iron death" in (Kobayashi et al., 2018) heart injury. and targeted intervention of iron death can effectively prevent the occurrence and development of heart disease.

The results of this experiment are consistent with the previous results. It is found that the electrocardiogram of AMI rats is abnormal and the ST segment is significantly increased, but electroacupuncture Shenmen (HT7) can significantly improve the changes of ST segment of ECG, which proves that electroacupuncture has important value in the treatment of ischemic heart disease.

Studies have shown that after myocardial injury, the level of Fe^{2+} in local tissue increases, which promotes the production of oxidative free radicals through fenton reaction, which promotes lipid peroxidation and leads to "ferroptosis" in surrounding tissues and cells (Dixon et al., 2012). As a major cofactor, GSH inhibits glutathione peroxidase (GPX4) and directly leads to the accumulation of lipid peroxides and activation of "ferroptosis". The results showed that compared with the sham operation group, the iron content in the myocardial tissue of the model group increased and the activity of GSH decreased（P<0.01）, while the iron content of the myocardial tissue decreased and the activity of GSH increased in the electroacupuncture group compared with the model group（P<0.01）. (Xie et al., 2016) Iron is a necessary trace element for life, which exists mainly in the form of bivalent and trivalent iron ions in the body. Ferrous ions (Fe^{2+}) are oxidized to trivalent iron ions (Fe^{3+}) by ceruloplasmin (ceruloplasmin,CP) and bound to membrane transferrin (transferrin,TF) through membrane protein transferrin receptor 1 (transferrin receptor protein1. Ferrous ions (Fe^{2+}) are oxidized to trivalent iron ions (Fe^{3+}) by ceruloplasmin (ceruloplasmin) and membrane transferrin (transferrin,TF) TFR1) forms a complex to transport iron ions into the cell by endocytosis for the body to use. After satisfying the iron needed for body metabolism, the excess iron will be quickly absorbed by the liver and stored by binding with ferritin light chain polypeptide (ferritin light chain,FTL) and ferritin heavy chain polypeptide (ferritin heavy chain 1) to avoid oxidative damage to the body caused by excessive iron in circulation. It has been found that (Manz et al., 2016) inhibits the expression of transferrin receptor (TfR1), the main transcriptional factor of iron metabolism, iron response element binding protein 2-(iron response element binding protein-2 (IREB2). The expression of ferritin light chain polypeptide (FTL) and ferritin heavy chain polypeptide 1 (FTH1) in ferritin subunit increased significantly. Therefore, we know that ferritin regulates the homeostasis of iron metabolism is an important regulating point in the mechanism of "ferroptosis". Long chain acyl-CoA synthetase 4 (ACSL4) (Yuan et al., 2016) participates in the synthesis of negatively charged membrane phospholipids such as phosphatidylethanolamine or phosphatidylinositol, and is an important gene in the pathway of lipid metabolism. Studies have shown that (Doll et al., 2017) can effectively reduce the rate of "ferroptosis" induced by erastin and RSL3 by knockout ACSL4 gene in mouse and human cells, which is a key factor in the implementation of "ferroptosis". Glutathione peroxidase 4 (glutathione peroxidase4, GPX4), as a selenoprotein for repairing oxidative damage of lipid cells in mammals, protects the structure and function of cell membrane from the damage and interference

of peroxides. It has been found that (Yang et al., 2014) knockout of mouse membrane lipid repair enzyme-GPX4 or direct use of GPX4 inhibitors can promote lipid peroxidation and intracellular reactive oxygen species accumulation, and GPX4 may be the key regulator of iron death. In this experiment, we found that the protein expression levels of GPX4,

FTH1mRNA, GPX4 and FTH1 in the model group were significantly lower than those in the sham operation group（P<0.01）, while the protein expression levels of TfR1, ACSL4 mRNA, TfR1 and ACSL4 in the model group were significantly higher than those in the model group（P<0.01）, while the protein expression levels of GPX4, FTH1mRNA, GPX4 and FTH1 in the electroacupuncture group were significantly higher than those in the model group（P<0.01）, while the protein expression levels of TfR1, ACSL4 mRNA, TFR1 and ACSL4 in the electroacupuncture group were significantly lower than those in the model group（P<0.01）. All these suggest that electroacupuncture "Shenmen" (HT7) can regulate the "ferroptosis" of cardiomyocytes induced by ischemic heart disease, and GPX4, FTH1, TfR1 and ACSL4 play an important role in its occurrence and development. Under the condition of ischemia and hypoxia, iron homeostasis regulation pathway was activated, iron ion accumulation and reactive oxygen species production increased, TfR1 and ACSL4 expression increased, GPX4 and FTH1 expression decreased, which induced "iron death" of cardiomyocytes. Electroacupuncture may inhibit cardiomyocyte "iron death", prevent cell death and achieve myocardial protection, but the related mechanism needs to be further studied.

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