

Original Article

Protective effect of restructuring klotho protein combined with trimetazidine on myocardial injury in mice

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Abstract: Objective: To study the protective effect of klotho protein combined with trimetazidine on the heart diseases in mice. Methods: A total of sixty mice were randomly divided into five groups as follow: N.S group (placebo group), ISO group (injury group), KL group, TMZ group and KL&TMZ group. KL group, TMZ group and KL&TMZ group were therapeutic groups. The difference was calculated according to the index of cardiac effort (HW/BW), the levels LDH, MDA, SOD and concentrations of Klotho in plasma. The cardiac muscular tissue was performed histopathologically. Results: The experiment models built for the requirements. HW/BW, LDH and MDA of Treatment groups were significantly lower than that of ISO group, SOD and concentrations of klotho were significantly higher than that of ISO group. The heart size of ISO group was obviously larger than that of N.S group. HE dyed results showed that the diameter of myocardial cell in ISO group was obviously larger than that of N.S group, combined with muscle fibers disorder myocardial hyperplastic and interstitial fibroblasts. The changes in treatment groups was much smaller than that of ISO group. The tan dye distribution of the treatment groups was significantly lower than that of ISO group showed by Caspase 3 dyed. Color positive results showed obvious practical color distribution. The difference was particularly evident in klotho protein combined with TMZ group. Conclusion: Restructuring of klotho protein combined with TMZ had protective effect on myocardial injury in mice.

Keywords: Klotho protein, trimetazidine, myocardial injury, isoproterenol, mice

Introduction

Recently China faces serious aged problem. The incidence of cardiovascular diseases has reached 290 million and it is the most common cause of death in China. Any changes of the structure and function caused by the initial myocardial injury, all can lead to low ventricular function and cause heart failure. The occurrence and development of ventricular remodeling caused by various heart diseases, is now recognized as the root cause of Terminal stage of chronic heart failure. With the progress of clinical medical therapy, such as coronary angiography and stent implantation as well as the development of coronary artery bypass graft surgery, remarkable progress has been made in rescuing the dying myocardium and inhibiting ventricular remodeling. Hower these measures can not completely reverse the deterioration of

the ventricular remodeling and improve the clinical symptoms of heart failure. Therefore, seek the key molecules and drug to reverse the pathological changes to reduce the incidence of heart failure and sudden death is of great significance. In this study, isoproterenol (ISO) stress was used to establish the model of myocardial injury in mice, serum lactate dehydrogenase (LDH) activity, malondialdehyde (MAD), superoxide dismutase (SOD) and concentrations of Klotho in plasma were used as the index of myocardial injury, paraffin section, HE staining, Massion dyeing and Caspase 3 dying were used as histopathological methods. This study show that klotho protein combine trimetazidine hydrochloride (TMZ) can prevent myocardium from damage and to analyze its possible mechanism. We look forward to providing a new approach for the treatment of cardiovascular diseases.

Table 1. The comparison of N.S group and ISO group ($\bar{x} \pm s$)

Group	N.S	ISO
HW/BW	0.42 ± 0.02	0.50 ± 0.03**
SOD activity (U/ml)	30.55 ± 7.02	18.32 ± 3.88**
LDH activity (U/L)	6260.73 ± 661.29	15834.98 ± 2372.68**
MDA content (nmol/ml)	5.23 ± 2.37	12.76 ± 6.66**

Note: compared with the levels in N.S group, **P<0.01.

and at the same time, intraperitoneal injection of Klotho proteins 0.01 mg/kg.d, once daily. (5) TMZ&KL group: as the ISO group, and at the same time, gastric infusion of trimetazidine 48 mg/kg.d, twice daily and intraperitoneal injection of Klotho proteins 0.01 mg/kg.d, once daily.

Materials and methods

Materials

Experimental healthy SPF inbred BalB/C 60 mice (provided by experimental animal center of Xi'an Jiaotong University Health Science Center, SYXK (shan) 2016-03-08-1247). Isopropyl epinephrine injection: Shanghai Tianfeng Pharmaceutical Co., LTD., batch number: 411-50102. Trimetazidine hydrochloride: servicer (Tianjin) Pharmaceutical Co., LTD., Batch number: 2008262. Klotho protein: 100 mg, Cloud-Clone Corp, American. Klotho protein kits (Batch: 1606031), Determination of total superoxide dismutase (SOD) kit method (WST-1) (aBatch 20160612), Determination of malondialdehyde (MDA) kit (associates) (Batch 2016-0612), LDH kit (micro plate method) (Batch: 20160628) are supplied by Nanjing Jiancheng Bioengineering Institute; The refrigerated centrifuge (Eppendorf 5415R, New York, USA); Enzyme standard instrument. Electronic analytical balance (Meiteler, GERMANY). BX-60-optical microscope (Olympus, JAPAN). Spectrophotometer (Germany FLUOstar OPTIMA).

Research methods

Male BalB/C mice (6to7 weeks old, about 17 g), 60 in total, were randomly divided into 5 groups: (1) N.S group had two subgroups: N.S group 1: subcutaneous and intraperitoneal injection of N.S 10 ml/kg.d once daily; N.S group 2: subcutaneous injection of N.S 10 ml/kg.d, once daily and gastric infusion N.S 10 ml/kg.d, twice daily. (2) ISO group had two subgroups: ISO group 1: subcutaneous injection of ISO 5 mg/kg.d once daily and intraperitoneal injection of N.S 10 ml/kg.d, once daily. ISO group 2: subcutaneous injection of ISO 5 mg/kg.d once daily and gastric infusion of N.S 10 ml/kg.d, twice daily. (3) TMZ group: as the ISO group, and at the same time, gastric infusion trimetazidine 48 mg/kg.d, twice daily. (4) KL group: as the ISO group,

All of the treatments were given for nine days. On the 10th day of the total experimental time all mice were weighed and performed. Then the blood and heart tissues were sent to laboratory for biochemical and histopathological examination in formaldehyde solution.

Blood was stored in EP tube for 30 min, after put in centrifuge with the centrifugal parameters of 4°C, 1500×g for 15 min, the centrifugal serum placed in the refrigerator to 20 degrees, after the detection of plasma LDH activity and MDA content, SOD level, serum concentration of Klotho. The heart of mice was taken out and cut around of it, its residual liquid was taken by filter paper after brining, weighed it and calculated cardiac index percentage (HW/BW). Then put heart tissue in 4% paraformaldehyde fixed, doing routine paraffin section, HE staining, Massion and Caspase 3 dyeing.

Statistical analysis

All values were expressed as mean ± standard deviation ($\bar{x} \pm s$). The comparisons of the level of MDA, SOD, LDH and HW/BW between N.S group and ISO group were made by using Student T-test. The P values of <0.05 were considered statistically significant. The same processing factors between groups of data comparison using single factor analysis of variance; Not neat or do not conform to the normal distribution of variance by non-parametric test: difference was statistically significant (P<0.05). Analyses were performed using commercial software SPSS18.0/PC.

Result

Animal models conform to the requirements of the experiments

This research adopts the classic ISO induced experimental acute mice myocardial injury models. To assess the effectiveness of the experimental models, the experiment intro-

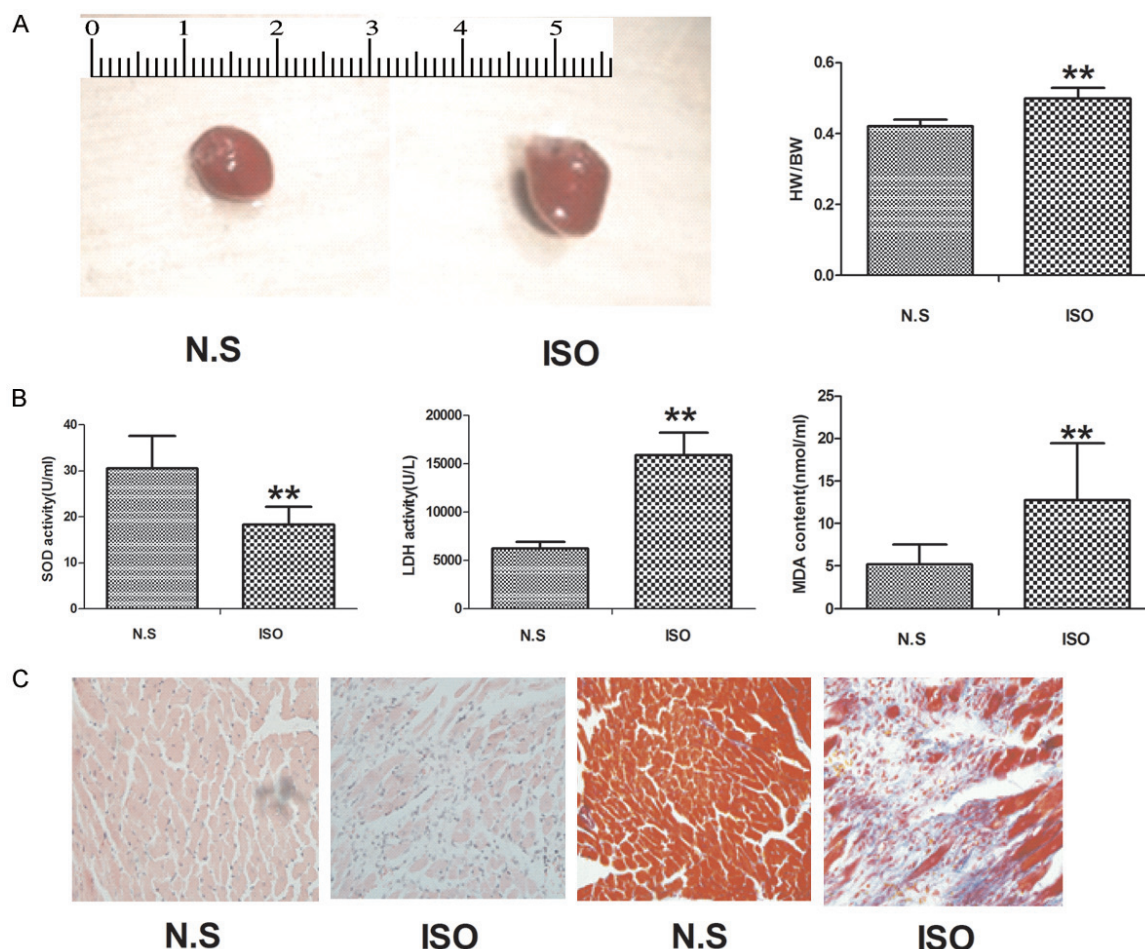


Figure 1. A. The comparison of heart size and weight in N.S group and ISO group (** $P < 0.01$). B. plasma LDH activity, MDA content and SOD level in N.S group and ISO group (** $P < 0.01$). C. Cardiac structural after HE dyed and Massion dyed in N.S group and ISO group.

Table 2. HW/BW, LDH, SOD, MDA and concentration of klotho in four groups ($\bar{x} \pm s$)

Group	HW/BW (%)	SOD (U/ml)	LDH (U/L)	MDA (nmol/ml)	Concentration of klotho (pg/ml)
ISO	0.50 ± 0.03	18.32 ± 3.89	12818.48 ± 3338.26	12.76 ± 6.65	151.31 ± 74.97
KL	$0.45 \pm 0.01^{**}$	$26.73 \pm 5.84^{**}$	$10090.21 \pm 1435.85^{**}$	$5.78 \pm 1.97^{**}$	$428.70 \pm 106.56^{**,\#}$
TMZ	$0.46 \pm 0.04^{**}$	$23.22 \pm 4.82^{*}$	$7911.99 \pm 2810.54^{**}$	$7.15 \pm 4.23^{*}$	$310.73 \pm 116.22^{**,\dagger}$
KL+TMZ	$0.43 \pm 0.01^{**,\#}$	$30.23 \pm 7.34^{**,\#}$	$8466.45 \pm 2035.93^{**,\dagger,\#}$	$4.74 \pm 3.35^{**}$	$440.29 \pm 163.03^{**,\#}$

Note: Compared with ISO group, * $P < 0.05$, ** $P < 0.01$; Compared with TMZ group * $P < 0.05$; Compared with KL group $^{\dagger}P < 0.05$.

duced in HW/BW % index, LDH activity, MDA content, SOD level of detection index and using HE, Massion coloring and establishes N.S control group. Compared with N.S group, ISO group HW/BW % index, LDH activity, MDA content were significantly increased, SOD activity significantly reduced (Table 1; Figure 1A and 1B). HE staining results found that continuous sub-

cutaneous injection of ISO (9 days) can cause myocardial cell hypertrophy and muscle fibers arranged disorder (Figure 1C); Masson coloring found ISO can significantly increase myocardial interstitial and hemal wall collagen surrounding content and cause myocardial fibrosis (Figure 1C). Successful experimental model established.

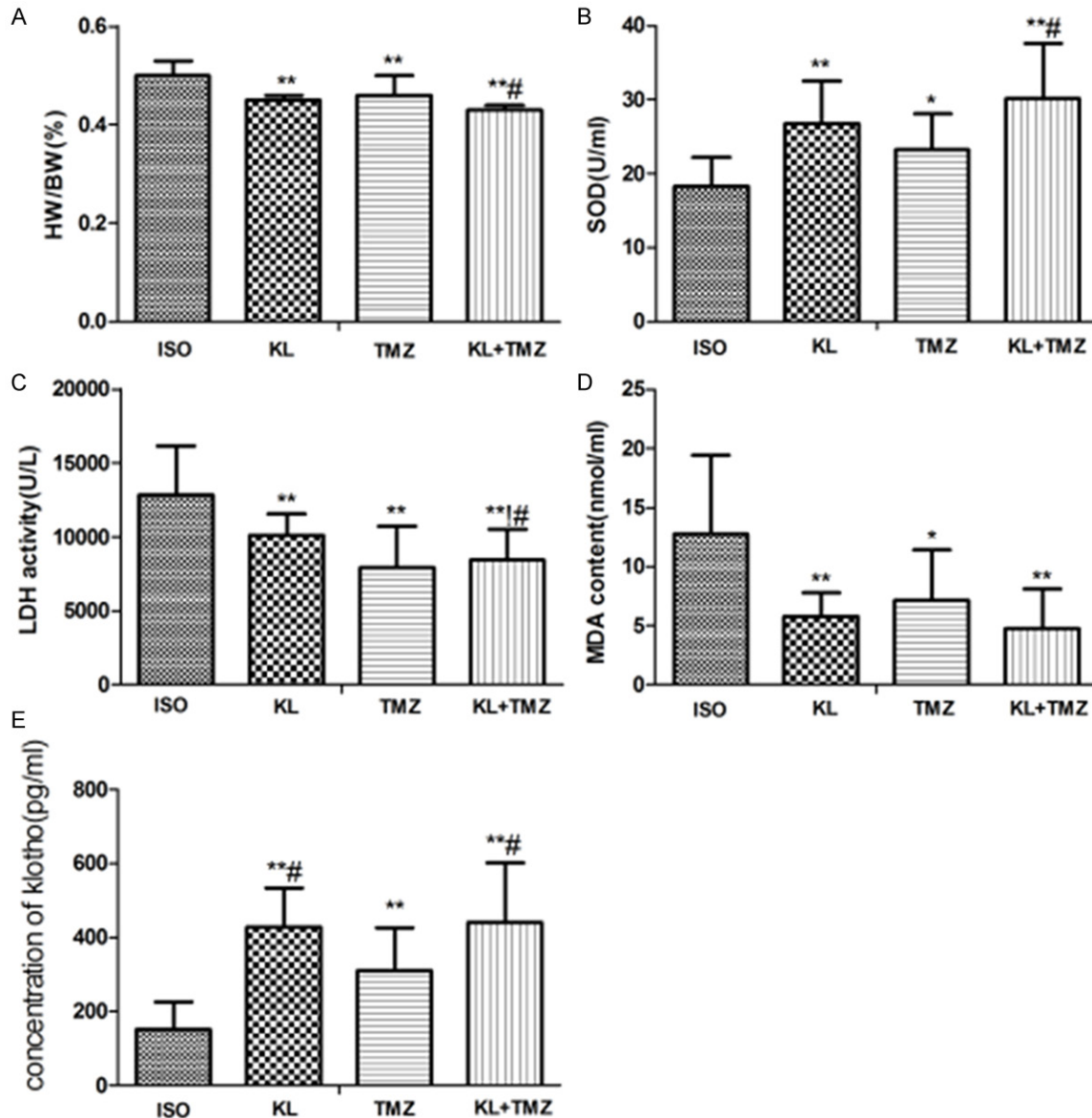


Figure 2. A. Comparison of HW/BW between KL group, TMZ group and KL&TMZ group (Compared with ISO group, *P < 0.05, **P < 0.01; Compared with TMZ group #P < 0.05); B. Comparison of SOD activity between KL group, TMZ group and KL&TMZ group (Compared with ISO group, *P < 0.05, **P < 0.01; Compared with TMZ group #P < 0.05); C. Comparison of LDH level between KL group, TMZ group and KL&TMZ group SOD (Compared with ISO group, *P < 0.05, **P < 0.01; Compared with TMZ group #P < 0.05; Compared with KL group !P < 0.05); D. Comparison of MDA content between KL group, TMZ group and KL&TMZ group (Compared with ISO group, *P < 0.05, **P < 0.01); E. Comparison of the concentration of klotho between KL group, TMZ group and KL&TMZ group SOD (Compared with ISO group, *P < 0.05, **P < 0.01; Compared with TMZ group #P < 0.05; Compared with KL group !P < 0.05).

Comparison of HW/BW, LDH, SOD, MDA and the concentration of klotho between KL group, TMZ group and KL&TMZ group

See **Table 2**. HW/BW % of KL group, TMZ group and KL&TMZ group were significantly lower than that of ISO group (P<0.01) (**Figure 2A**). SOD level of KL group, TMZ group and KL&TMZ

group were significantly lower than that of ISO group (P<0.01); SOD level of KL&TMZ group were significantly higher than that of TMZ group (P<0.01) (**Figure 2B**). LDH level of KL group, TMZ group and KL&TMZ group was lower than that of ISO group, the difference was statistically significant (P<0.01); LDH level of KL&TMZ group were significantly higher than that of TMZ

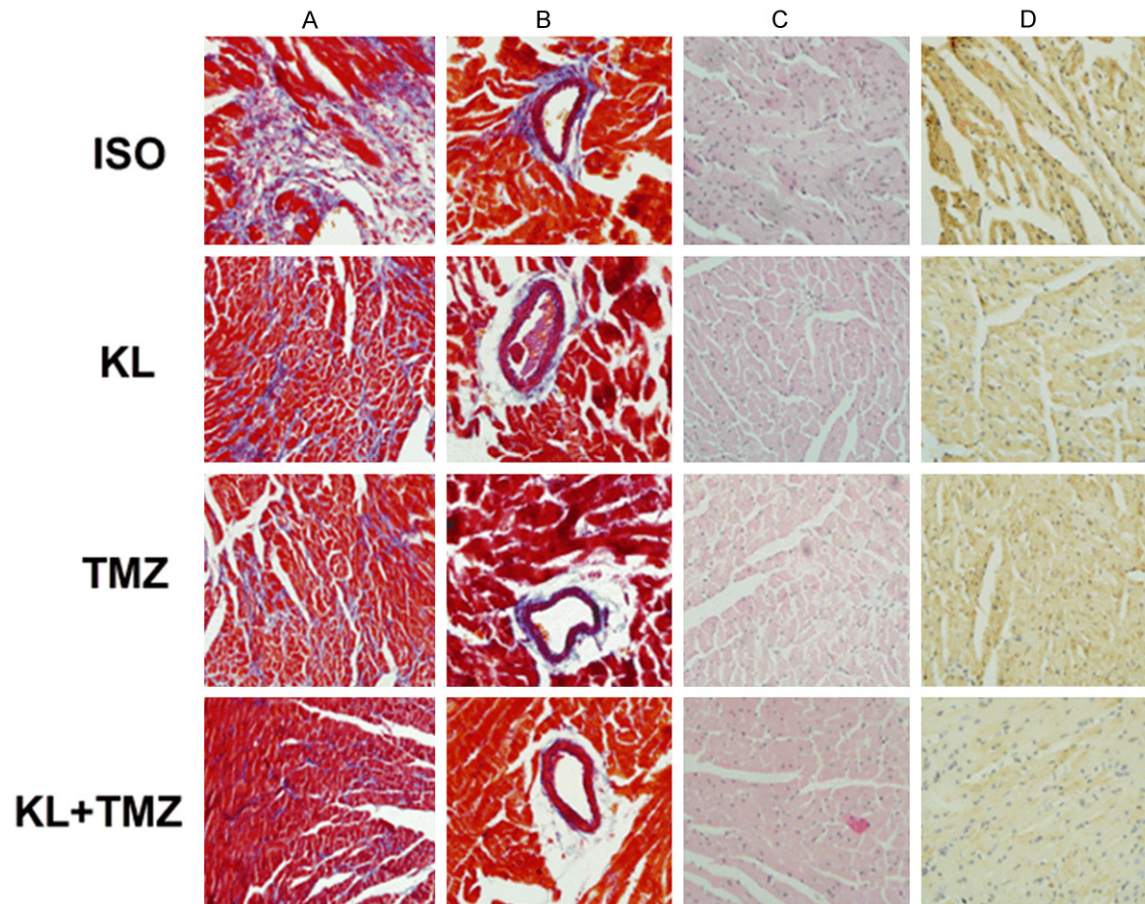


Figure 3. Staining results in different groups. A: Myocardial tissue of ISO, KL, TMZ group, KL&TMZ group by Massion dying; B: Myocardial interstitial and around the blood vessels of ISO, KL, TMZ group, KL&TMZ group by Massion dying; C: Myocardial tissue of ISO, KL, TMZ, KL&TMZ group by HE dying; D: Myocardial tissue of ISO, KL, TMZ group, KL&TMZ group by Caspase 3 dying.

group and were significantly lower than that of KL group ($P<0.01$) (**Figure 2C**). MDA content of TMZ group was significantly lower than that of ISO group, the difference between them was statistically significant ($P<0.05$); MDA content of KL group and KL&TMZ group was significantly lower than that of ISO group, the difference between them was statistically significant ($P<0.01$) (**Figure 2D**). Klotho concentration of KL group, TMZ group and KL&TMZ group was significantly lower than that of ISO group, the difference was statistically significant ($P<0.01$); klotho concentration of KL group and KL&TMZ group was significantly higher than that of TMZ group ($P<0.05$) (**Figure 2E**).

Cardiac muscle HE, Massion and Casepase 3 dyed in groups

HE dyed showed that the diameter of cardiomyocytes of mice in ISO group was significantly

greater than that of treatment groups, and with obvious muscle fibers disorder myocardial hyperplasia and interstitial fibrosis. The diameter of myocardial cells in was significantly small than that of ISO group, the degree of fibrous hyperplasia of TMZ group was significantly light. It show that there were a lot of collagen fiber distribution in myocardial interstitial and hemal wall in ISO group, and the number of myocardial interstitial collagen fibers and blood vessels decreased significantly in treatment groups by using Massion dying. The difference was particularly evident in klotho protein combined with TMZ group. There were no obviously difference between KL group and TMZ group. It results show that tan color distribution of treatment groups decreased significantly than that of ISO group by using Caspase 3 color (dyed positive results show the cell awards had large brownish yellow color distribution) (**Figure 3**).

Discussion

ISO is a strong beta agonist, it has the effect of positive isotropic shrinkage, and increase the heart rate, myocardial contraction force, myocardial oxygen consumption and accelerates conduction, as the result can cause cardiac critical load, myocardial microcirculation disorder and coronary spasm, myocardial necrosis and even sudden death. These pathological damage may be related to the mitochondrial energy metabolism disorder, the activation of apoptosis gene, oxidative stress and so on [1-3]. We know that the body produces oxygen free radicals through the enzyme system and non-enzymatic system. The amount of MDA who can damage biofilm often be reflected in the extent of lipid peroxidation. SOD can remove lipid peroxides, plays a vital role for reducing lipid peroxidation by regulating the balance of body's oxidation and antioxidant, its height can indirectly reflect the ability of the body resistance to oxidative damage. This experimental observed that myocardial free radical metabolism was disorder after mice ISO model building, MDA level elevated and SOD level decreased, LDH level and HW/BW index increased, myocardial cell and myofibril got degeneration and coagulation necrosis in the aspect of pathological examination compared with the control group.

Klotho is a newly anti-aging effect gene [4], exist mainly in the kidneys and brain tissue, it can produce two materials through gene expression, and they are protein membrane receptor and humoral regulation factor [5]. Studies [6] show that the Klotho gene had anti-oxidative stress, reduced vascular endothelial cell apoptosis, and protected endothelial cells [7-9], affected cell signal transduction pathways [10] and so on. These effects can prevent and control cardiovascular disease according to the pathogenesis of diseases, and improve the long-term outcome [11]. TMZ is a metabolic therapy for myocardial cell protection [12], it promote glucose metabolism by inhibiting fatty acid beta oxidation, and improve myocardial energy metabolism. After the success of the myocardial damage models, this study observed the protection effect of Klotho protein combined with TMZ on it. The oxidative stress state of myocardial tissue changed significantly, performance in SOD activity improved, LDH activity and MDA content decreased, collagen content of myocardial interstitial cells reduced, cardiac

weight index fell. Above show that restructure klotho protein had protective effect to mice myocardial injury, had the effect of clearing oxygen free radical and the effect of apoptosis resistance to prevent myocardial hypertrophy, it might be one of the mechanism reversing ventricular remodeling.

TMZ can raise klotho gene expression and alleviate myocardial damage, this study further observation results show that TMZ could increase blood klotho level. The correlation analysis show that it was negative correlation significantly between klotho and LDH or MDA, and it was positively correlated between klotho and SOD. It prompted that klotho protein was closely related to the body's antioxidant [13].

During this experiment, the serum LDH, MDA, cardiac index decreased and SOD improved significantly in combined treatment group after ISO induced myocardial injury, also found that muscle fibers disorder and myocardial interstitial fibroblasts hyperplasia decreased, the fiber numbers of myocardial interstitial collagen and blood vessels reduced. Klotho joint SMZ had improved protection and antioxidant effect obviously to ISO myocardial damage than Klotho recombinant proteins and TMZ alone, and it provided a certain theoretical basis for clinic therapy.

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Disclosure of conflict of interest

None.

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