Journal of Trace Elements in Medicine and Biology 56 (2019) 21–30

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

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

Epidemiology

Effects of selenium deficiency and low protein intake on the apoptosis through a mitochondria-dependent pathway

Liwei Zhang, Yanhui Gao, Hongqi Feng, Ning Zou, Kewei Wang, Dianjun Sun*

Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, Key Lab of Etiology and Epidemiology, National Health and Family Planning Commission (23618504), Harbin, 150086, Heilongjiang, China

ARTICLE INFO

Keywords:
Selenium deficiency
Low protein diet
Cardiacdysfunction
Oxidative stress
Apoptosis
Mitochondrial pathway

ABSTRACT

Objective: Selenium(Se)is an important trace element for human health. Studies have shown that selenium deficiency and low protein(Pr) intake are the primary risk factors for Keshan disease.The relationship between the cardiac malfunction induced by these two risk factors and the mitochondria-mediated apoptotic pathway is poorly understood.This study aimed to determine the effect of selenium deficiency and low protein intake on the mitochondria-mediated apoptotic pathway.

Methods: In the present study, 120 weaning Wistar rats were randomly fed one of six different diets. The myocardial tissue sections were deparaffinized in water and subjected to hematoxylin-eosin staining. Mitochondrial changes in the myocardial tissue were observed and photographed using an H-7650 Hitachi transmission electron microscope. Levels of whole blood Se were measured using hydride generation atomic fluorescence spectrometry. Whole blood glutathione peroxidase (GSH-Px) activity was measured using a glutathione peroxidase cellular activity assay kit. Malondialdehyde (MDA), total-anti-oxidizing-capability(T-AOC) and reactive oxygen species(ROS)levels in serum and myocardial tissue were measured using MDA, T-AOC and ROS kits. Apoptosis was detected by immunohistochemistry.

Results: Experimental results showed that the selenium-deficient diet decreased serum selenium levels and GSH-PX activity, which caused severe cardiac dysfunction. Importantly, the levels of MDA and ROS in serum and myocardial tissue defects were significantly increased, where as total-anti-oxidizing-capability(T-AOC) levels were dramatically decreased as a result of the combination of selenium deficiency and low protein intake ($P < 0.05$).The levels of cleaved caspase-9 and cleaved caspase-3 were enhanced, but the expression of B-cell lymphoma-2 (Bcl-2) was reduced ($P < 0.05$).

Conclusions: Our results suggest that selenium deficiency and low protein intake can cause oxidative stress in the myocardium and induce cell apoptosis via the mitochondria-mediated pathway.

1. Introduction

Selenium is an important component of glutathione peroxidase (GSH-Px) and a trace element that is important for human health. Selenium deficiency is closely linked to many diseases, such as Keshan disease [1]. Dietary selenium deficiency and low protein intake are the primary risk factors for Keshan disease [2–7], and the combined effects of the two factors are more harmful than a single factor alone [8].The early pathological changes associated with Keshan disease are abnormal structure and function of cardiac mitochondria. The combination of selenium deficiency and low protein intake causes mitochondrial damage due to the low activity of GSH-Px and the accumulation of ROS in the cell [9].The activity and content of GSH-Px are important indicators that are used in biological studies to measure selenium levels [10]. Mitochondrial initiation of endogenous pathway was found in myocardial tissue in autopsy of acute, subacute and chronic Keshan disease, which is closely related to selenium deficiency [11].

The mitochondria-mediated apoptotic pathway plays a key role in maintaining a balance between cell proliferation and death. When the

Abbreviations: Se, selenium; Pr, protein; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; ROS, reactive oxygen species; T-AOC, total-anti-oxidizing-capability; Bcl-2, B-cell lymphoma-2

* Corresponding author at: Center for Endemic Disease Control, Chinese Center for Disease Control and Prevention, Harbin Medical University, 157 Baojian Road, Harbin, 150081, China.

E-mail addresses: zhangliwei@ems.hrbmu.edu.cn (L. Zhang), gaoyanhui@hrbmu.edu.cn (Y. Gao), fenghongqi@ems.hrbmu.edu.cn (H. Feng), zouning@hrbmu.edu.cn (N. Zou), wangkewei@hrbmu.edu.cn (K. Wang), hrbmusdj@hrbmu.edu.cn (D. Sun).

https://doi.org/10.1016/j.jtemb.2019.06.019
Received 2 January 2019; Received in revised form 14 April 2019; Accepted 21 June 2019

balance is disturbed as a result of selenium deficiency and low protein intake, the mitochondria-mediated pathway are activated. In this study, we compared the level of cleaved caspase-9,cleaved caspase-3, and Bcl-2 in the mitochondrial pathway and further explored the effects of selenium deficiency and a low protein diet in rats.

2. Materials and methods

2.1. Animals and experimental groups

The animal procedures and subsequent experiment described in this study were approved by the Institutional Animal Use and Care Committee of Harbin Medical Sciences University. One hundred and twenty weaning male Wistar rats (SPF class, 18days old, body weight 78 ± 2 g) were purchased from Beijing Vital River Laboratory Animal Technology Co.,Ltd. The rats were randomly divided into six groups. They had free access to demineralized water and were kept under environmental conditions (12 h light-darkcycle, temperature 22 ± 2 °C, humidity 65 ± 4%).The six treatment groups were fed for 90 days, and all of the diets were purchased from Beijing Keao Xieli Feed Co.,Ltd., according to theAIN-93 M formula [12].The control group and the doxorubicin group were fed a standard diet according to the AIN-93 G formula [13,14]. The other groups were fed a corresponding diet, as shown in Table 1.

2.2. Selenium status and glutathione peroxidase activity

Levels of whole blood Se were measured using hydride generation atomic fluorescence spectrometry. The whole blood samples were digested with concentrated nitric acid and perchloric acid in a 4:1 ratio. Thermal digestion was performed at 180 °C by using a digital KXS-3.6 electric sand bath (Lei Yun Test Instrument Manufacturing Co., Ltd., Shanghai, China). The calibration curves were prepared in a solution containing 6% (v/v) hydrochloric acid with different volumes of 1 μg/L Se standard solution (CAS#7782-49-2). Serum samples were determined by AFS-930 dual channel atomic fluorescence spectrometry (Jitian Instrument Co., Ltd., Beijing, China). Quality control (QC) was ensured by analyzing certified reference material (GBW080193) from the 301 Hospital Clinical Research Institute and the Ministry of Geology and Minerals Rock and Minerals Laboratory.

Whole blood GSH-Px activity was measured using a glutathione peroxidase cellular activity assay kit (Nanjing Jian cheng Bioengineering Institute, China) according to the manufacturer's instructions.

Table 1
Ingredients of diet.

Group	Number	Selenium (mg/kg)	Protein(%)	other nutrients ingredients
Control	20	0.24	18.8	a
Deficiency selenium	20	0.01	18.8	a
Deficiency protein	20	0.24	10	a
Deficiency selenium and protein(se-pr)	20	0.01	10	a
Corn from Keshan disease area	20	0.01	7.2	b
Doxorubicin	20	0.24	18.8	a

Note: 1. Doxorubicin group : doxorubicin 2 mg/kg, intraperitoneal injection, once a week for 7 times.
2. a: Other nutrient ingredients: Corn Starch 39.7%, Casein-Vitamin Free 20.0%,Maltodextrin13.2%, Sucrose10.0%, Soybean Oil 7.0%, Powdered Cellulose 5.0%, AIN93 G Mineral Mix 3.5%, AIN 93 Vitamin Mix1.0%, L-Cystine0.3%, Choline Bitartrate0.25%, t-Butylhydroquinone 0.0014%.
b: Other nutrient ingredients: Starch 70%, unsaturated fatty acid52%, Mineral Mix 3.0%, Vitamin Mix1.0%, Lysine 0.27%, threonine 0.31%, methionine + cystine 0.31%.

2.3. Hematoxylin-eosin staining

The myocardial tissue sections were deparaffinized in water and subjected to hematoxylin-eosin staining as follows. Tissues were soaked in hematoxyl in stain for 3–5 min, tap water for 1 min, hydrochloric acid differentiation solution for 2 s (dipped twice), tap water for 5 min (back to blue), eosin stain for 2–3 min, and tap water for 30 s. Then, the tissue sections were dehydrated with 85% ethanol for 20 s, 95% hexanol I for 1 min, 95% ethanol II for 1 min, 100% ethanol I for 2 min., and 100% ethanol II for 2 min. The tissue sections were then transparentized via xylene I for 5 min and xylene II for 5 min. Finally, an eutral gum seal was applied. Myocardial tissue damage was observed and compared among the Se deficiency, low protein, and control groups. Photographs were taken using an OLYMPUS BX53 microscope (Olympus Corporation of Japan).

2.4. Transmission Electron microscopy of myocardial tissue

The cardiac apex (1.0 mm^3) was fixed in 2.5% glutaraldehyde and rinsed with 1 M phosphate buffer (pH = 7.2), then fixed with 1% osmium tetroxide for 1 h and stained with 1% uranyl acetate. Tissue was dehydrated by soaking in acetone (acetone to the embedding liquid = 1:137 °C, 2 h; acetone to the embedding liquid = 1:4, 37 °C, overnight; 100% of the embedding liquid,45 °C, 2 h) and embedded at 45 °C for 3 h and 65 °C for 48 h. Mitochondrial changes in the myocardial tissue of Se-deficient rats and normal controls were observed and photographed using an H-7650 Hitachi transmission electron microscope (Hitachi, Japan).

2.5. TUNEL analysis

Myocardial apoptosis was quantified using a terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit (Roche Diagnostics) according to the manufacturer's instructions. The ImagePro Plus image analysis software and 400× optical microscope images were analyzed to determine the cumulative optical density (IOD) of positive cells in each group for a semiquantitative analysis.

2.6. Levels of MDA, ROS and T-AOC in serum and myocardial tissue

Malondialdehyde (MDA), total-anti-oxidizing-capability (T-AOC) and reactive oxygen species (ROS) levels in serum and myocardial tissue were measured using MDA, T-AOC and ROS kits (Nanjing Institute of Bioengineering, China) according to the manufacturer's instructions.

2.7. Apoptosis was detected by immunohistochemistry

The sections were deparaffinized. Then, 3% H_2O_2 was added to inactivate endogenous peroxidases. Subsequently, 0.01 M/L citric acid was used to retrieval the antigen, and protein expression was detected by SABC immunohistochemistry (the SABC kit was purchased from Wuhan Bude Bioengineering Co., Ltd.). All of the sections were developed with DAB, stained with hematoxylin, and sealed with a neutral resin. In the negative control group, the primary antibody was replaced with PBS, and the other steps were the same as those mentioned previously. Images were analyzed using ImagePro Plus image analysis software and an optical microscope 400×, and brown-yellow particles in the cytoplasm or nucleus were used as positive criteria. The cumulative optical density (IOD) of positive signals in each group was measured, and a semiquantitative analysis was performed.

2.8. Statistical analysis

In immunohistochemistry and TUNEL images, brownish yellow is positively stained and the total amount of cumulative optical density

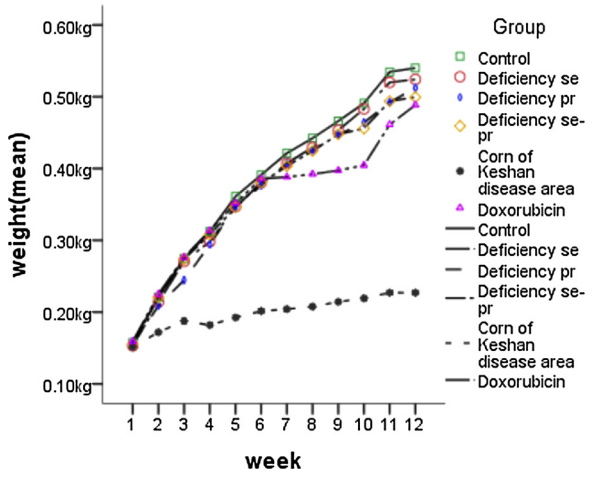

Fig. 1. Weight of each treatment group of rats.

values per unit area is compared between groups. The relative values of IOD SUM between the experimental group and the control group were compared. The data obtained is normally distributed, so we use one-way ANOVA to test the differences between the groups. Statistical analysis was performed using SPSS for Windows software (version 19; IBM, Armonk, NY, USA) and expressed as the mean ± SD. $P < 0.05$ is considered significant for all analyses.

3. Results

3.1. Comparison of body weight

The body weights of rats were measured once a week for12 weeks to evaluate the general nutritional status of experimental animals in different groups. The results showed that the bodyweights of rats fed with corn from Keshan disease area (corn groups) were significantly lower than rats in other groups from the second week. After sixth week, doxorubicin was injected, the body weight of rats were lower than other groups, but still higher than corn group. Except for these two groups, the body weights of rats in other treatment groups were lower than control rats in the last 6 weeks (Fig. 1).

3.2. Concentrations of blood Se and GSH-Px activity

Selenium concentrations in whole blood were measured to determine the effect of dietary selenium deficiency. The results showed that the levels of selenium in whole blood were significantly lower in the treatment groups than in the control group ($P < 0.05$) (Fig. 2).

Many human studies have estimated selenium status through the activity of GSH-P × . GSH-Px has been considered an important indicators since it is a selenoenzyme. Decreased GSH-Px activity was observed in whole blood of the Se-deficient rats ($p < 0.05$) (Fig. 3) compared to the GSH-Px activity in the control group. This finding indicates that the biosynthesis of GSH-Px was already affected to a large extent after rats were fed a selenium-deficient diet.

3.3. Hematoxylin-eosin staining

To determine whether the selenium-deficient diet had an effect on myocardial cell morphology, we stained the myocardial tissue with a hematoxylin-eosin staining solution to compare the morphological changes between the cardiomyocytes in the Se-deficient rats and those in the control group. In the control group, the myocardial fibers were arranged neatly, and the nucleus and cytoplasm were uniformly stained. Compared with the results from the control group, myocardial damage was increased in the selenium-deficient rats. In the treatment

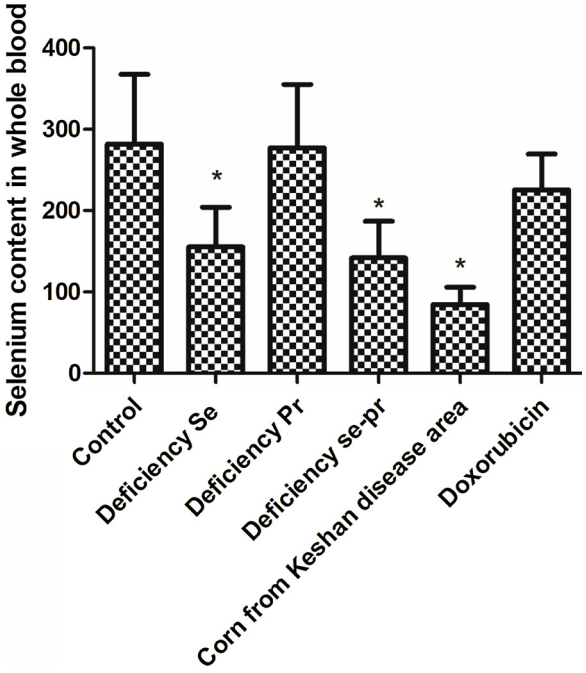

Fig. 2. Selenium content in whole blood of each treatment group of rats.

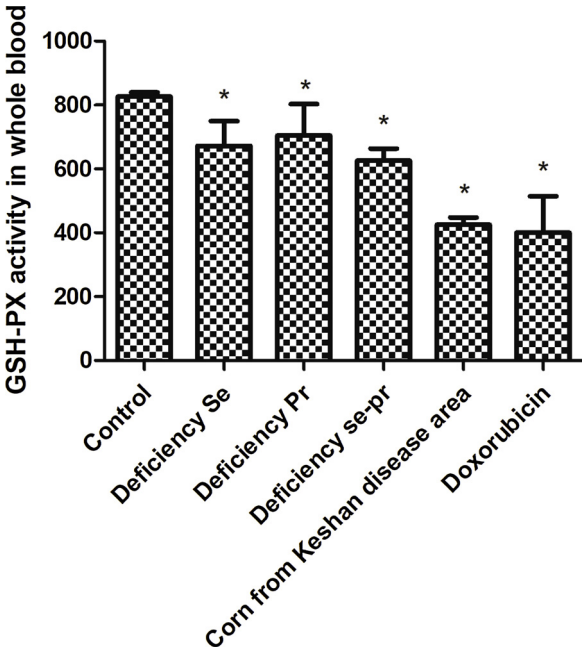

Fig. 3. GSH-PX activity in whole blood of each treatment group of rats.

group, parenchymal degeneration and necrosisac companied by inflammatory cell infiltration were observed in the endocardium and interventricular septum, and some cardiomyocytes were pyknotic and deeply stained. (Fig. 4).

3.4. Transmission electron microscopy of Myocardial tissue

To determine whether the selenium-deficient diet had an impact on the mitochondria in the myocardium, we compared the ultrastructural changes in Se-deficient rats with the ultrastructural changes in the normal controls. The mitochondria of the normal control rats showed a uniform distribution and good alignment of myofilaments. Most mitochondria in the selenium-deficient rats showed irregular swelling,

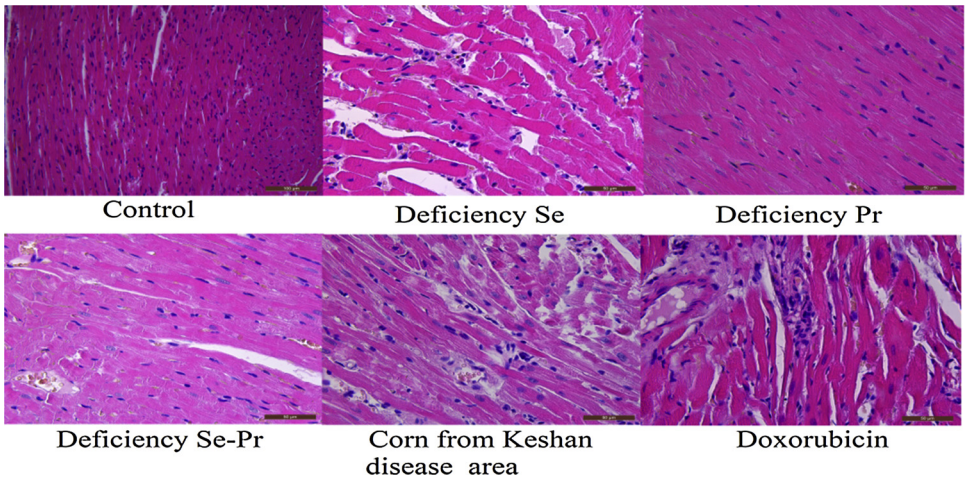

Fig. 4. Myocardial tissue from Wistar rats was used for HE staining (200x magnification). The control group, the myocardial fibers were arranged neatly, and the nucleus and cytoplasm were uniformly stained. Selenium-deficient group, there were some areas of cardiomyocytes degeneration, necrosis. The protein-deficient group, there were some areas of cardiomyocytes degeneration, necrosis, similar to selenium-deficient group. The selenium-deficient and low protein group, myocardial damage was increased, cardiomyocytes degeneration and necros were more serious. Corn from the Keshan disease area group and the doxorubicin group, myocardial damage were increased, cardiomyocytes degeneration and necros were most serious, parenchymal degeneration and necrosisac companied by inflammatory cell infiltration were observed in the endocardium and interventricular septum, and some cardiomyocytes were pyknotic and deeply stained.

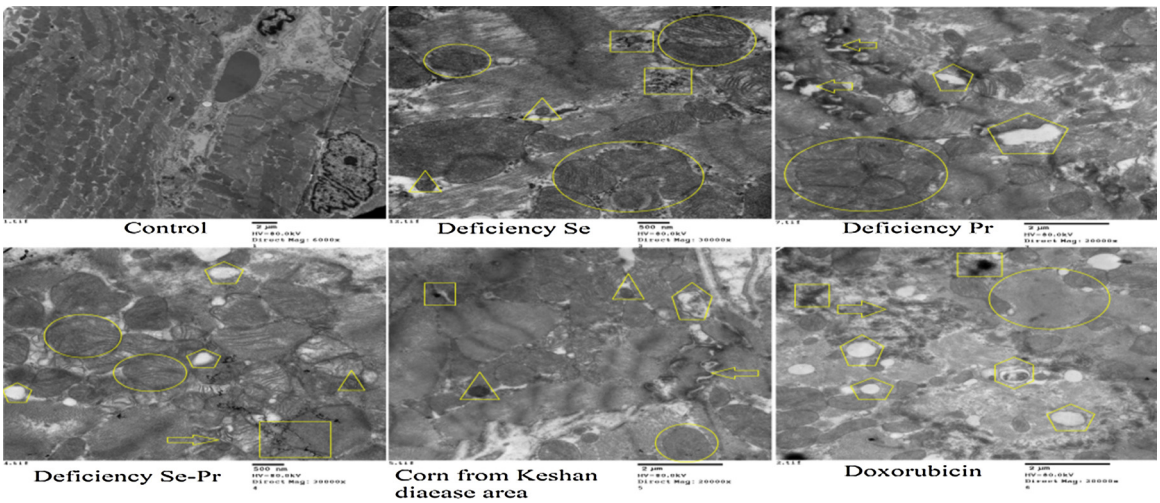

Fig. 5. Myocardial tissue from Wistar rats was used for transmission electron microscopy (200x magnification). The mitochondria of control group showed a uniform distribution and good alignment of myofilaments. The mitochondria of selenium-deficient group showed mitochondria swell, form autophagosomes, appear calcification. The protein-deficient group showed dissolve the disk, mitochondria swell, form autophagosomes, appear calcification, dissolve the disk, sarcoplasmic reticulum expansion. The selenium-deficient and low protein group showed more serious, there were more dissolve the disk, mitochondria swell, autophagosomes, calcification, dissolve the disk, sarcoplasmic reticulum expansion. Corn from the Keshan disease area and the doxorubicin group, mitochondria damage were most serious, showed irregular swelling, fractured myofilaments, sarcoplasmic reticulum expansion, a contraction band, multiple calcifications around the mitochondria, mitochondrial membrane rupture and lamellar body formation or myeloid changes.
Note: Circle: swollen mitochondria;single arrow: Intercalated disk dissolving; square: calcification point; triangle: autophagy lysosome; pentagon: sarcoplasmic reticulum expansion; hexagon: autophagosome.

fractured myofilaments, sarcoplasmic reticulum expansion, a contraction band, multiple calcifications around the mitochondria, mitochondrial membrane rupture and lamellar body formation or myeloid changes (Fig. 5).

3.5. Comparison of levels of MDA, ROS and T-AOC in serum and myocardial tissue

To determine whether the selenium-deficient diet had an impact on the myocardial oxidative defense system, we compared MDA, ROS and T-AOC in the serum and myocardial tissue of selenium-deficient rats and normal controls. The serum MDA and ROS levels were significantly higher in the treatment group than in the control group ($P < 0.05$).The level of serum T-AOC was significantly lower in the treatment group than in the control group ($P < 0.05$) (Figs. 6–8).The myocardial MDA level was significantly higher in the treatment groups than in the control group, with the exception of the selenium-deficient group ($P < 0.05$). The level of ROS in the myocardium was significantly higher in the treatment groups than in the control group, with the exception of the selenium-deficient group and the low protein group ($P < 0.05$). The level of myocardial T-AOC was significantly lower in the treatment group than in the control group ($P < 0.05$) (Figs. 9–11).

3.6. TUNEL assay

To determine whether the selenium-deficient diet had an impact on cardiomyocyte apoptosis, we used the TUNEL assay method to compare the apoptotic cell changes in Se-deficient rats and normal controls. The apoptotic cells were determined according to the optical density (OD) value of the slices from the myocardial tissue samples in different groups. Apoptotic cell were gradually increased and widely distributed in experimental groups, which brown-yellow staining was stronger, compared with control group.The OD value of apoptotic cells in control group (0.58 ± 0.14), the selenium-deficient group (0.66 ± 0.19), the

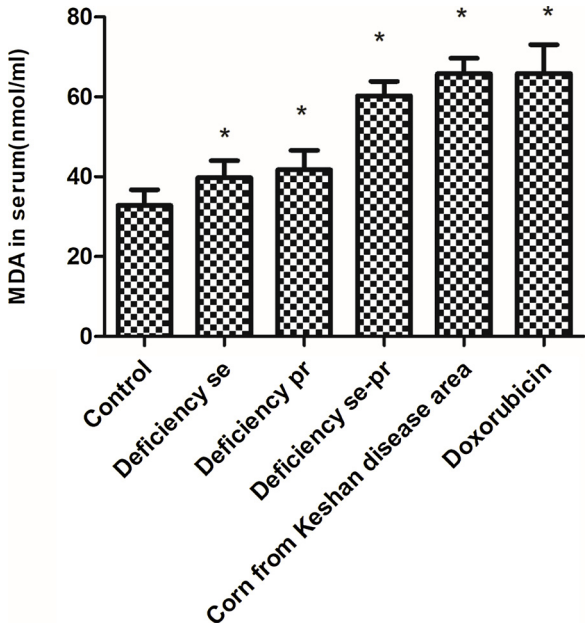

Fig. 6. MDA content in serum of each treatment group of rats.

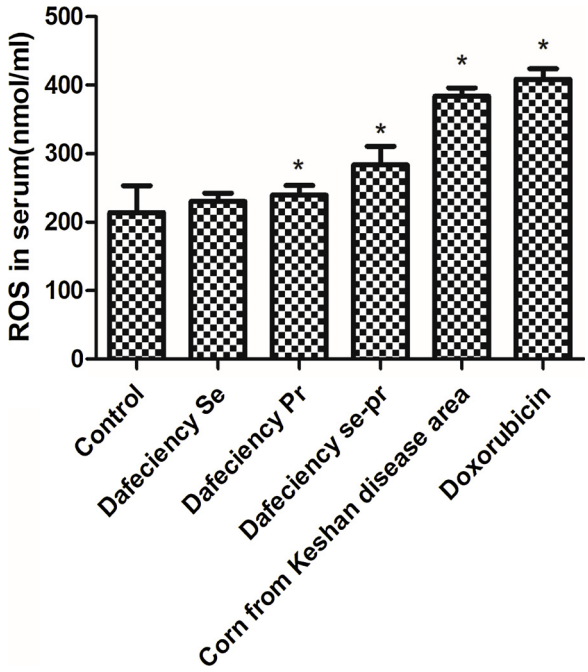

Fig. 7. ROS content in serum of each treatment group of rats.

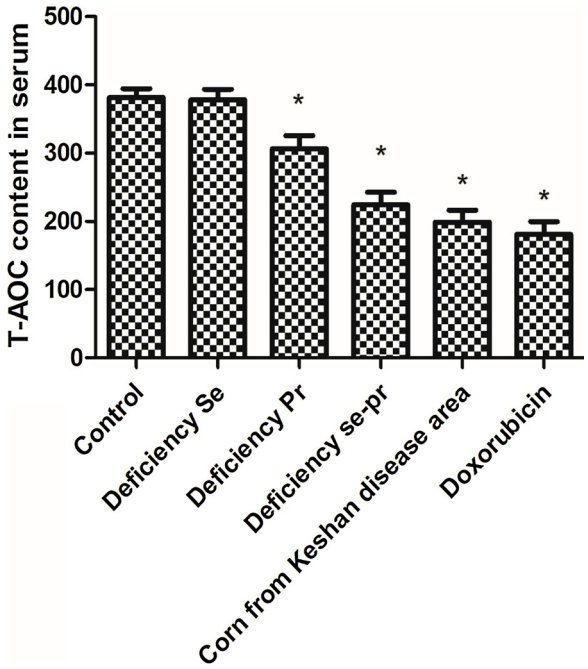

Fig. 8. T-AOC content in serum of each treatment group of rats.

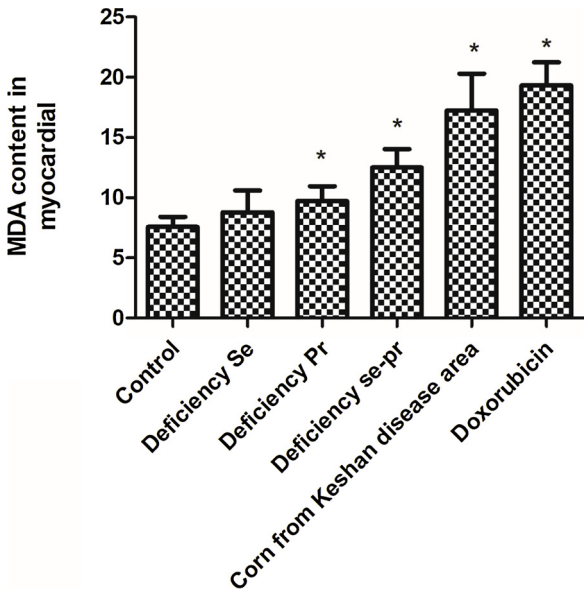

Fig. 9. MDA content in myocardial of each treatment group of rats.

low protein group (0.66 ± 0.18), the protein and selenium-deficient group (0.76 ± 0.19), corn from the Keshan disease area group (0.81 ± 0.17), the doxorubicin group (0.82 ± 0.22) ($P < 0.05$) (Fig. 12). The result in Fig. 13 shows the relative number of the OD value, which is the ratio of each OD value to control group.

3.7. Comparison of gene expression in the mitochondrial apoptotic pathway

The effect of a Se-deficient diet on gene expression in the mitochondrial apoptotic pathway was determined by comparing cleaved caspase-9, cleaved caspase-3, and Bcl-2 levels in the myocardial tissues of Se-deficient rats and normal controls. The differences in protein expression were determined according to the optical density (OD) value of the slices from the myocardial tissue samples in different groups. The expression of cleaved caspase-9 protein in Se-deficient group (7.39 ± 2.13), low protein group (7.70 ± 1.59), protein and selenium-deficient group (9.66 ± 1.97), corn from the Keshan disease area group (10.13 ± 1.88) and doxorubicin group (10.39 ± 2.18) were higher compared with control group (5.64 ± 0.94) (Fig. 14).The expression of cleaved caspase-3 protein in Se-deficient group (3.70 ± 0.52), low protein group (3.97 ± 0.69), protein and selenium-deficient group (5.75 ± 1.17), corn from the Keshan disease area group (7.30 ± 0.97) and doxorubicin group (7.84 ± 1.07) were higher compared with control group (2.96 ± 0.58) (Fig. 15).Compared with the control group (2.39 ± 0.32), the protein expression of Bcl-2 in Se-deficient group (2.15 ± 0.44), low protein group(1.92 ± 0.46), protein and selenium-deficient group (1.25 ± 0.48), corn from the Keshan disease area group (0.91 ± 0.35) and doxorubicin group (0.86 ± 0.29) gradually decreased (Fig. 16), ($P < 0.05$). The result in

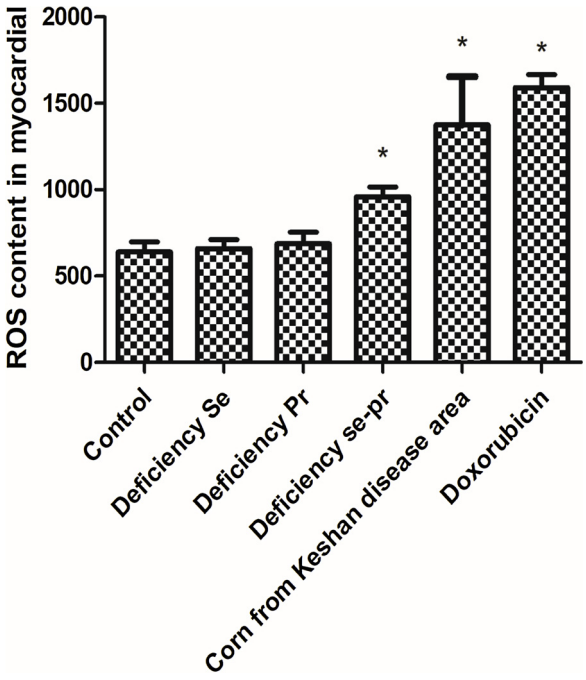

Fig. 10. ROS content in myocardial of each treatment group of rats.

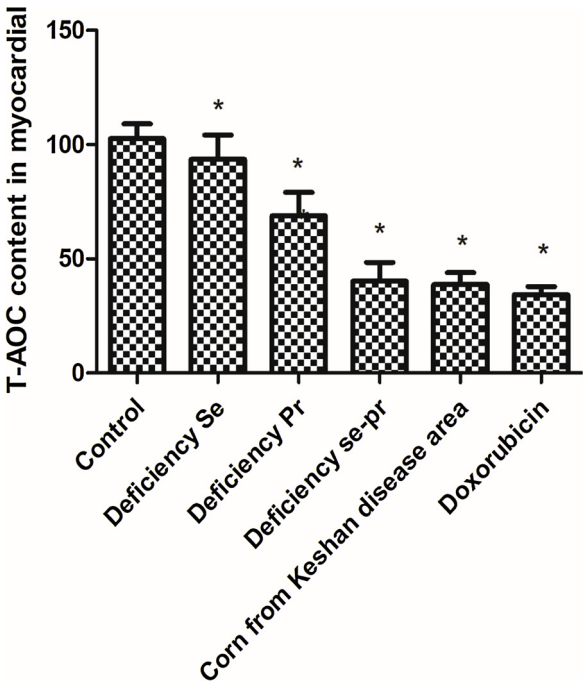

Fig. 11. T-AOC content in myocardial of each treatment group of rats.

Fig. 17 shows the relative number of the OD value, which is the ratio of each OD of value to control group.

4. Discussion

In this study, we investigated how selenium deficiency and a low protein diet affected the mitochondria-mediated apoptotic pathway, which is related to mitochondrial apoptosis and oxidative stress in Wistar rats. Our results suggest that selenium deficiency and low protein intake can induce pathological changes via the mitochondria-mediated apoptotic pathway. Combined selenium and protein deficiency has been implicated in the etiology of cardiovascular diseases, such as Keshan disease. Thus, the observed alterations might be early indicators of a predisposition to this disease.

Keshan disease occurs more frequently in a low-selenium zone from northeast to southwest China [15,16]. Chinese researchers have carried out in-depth research on the etiology and pathogenesis of Keshan disease and have proposed various hypotheses, such as single dietary factors, selenium deficiency, viral infection, etc. [17–19]; However, no single hypothesis can fully explain the cause of Keshan disease. It is currently believed that Keshan disease may be caused by multiple factors. Our previous studies have found that the susceptible residents live in a low selenium environment and have poor nutritional status without enough supplement of non-staple food [20]. Studies by Al-Rasheed and Chan have shown that a low selenium diet can aggravate heart damage [21,22]. Multiple studies, including a study by Zhang YC, have demonstrated that, in the case of low selenium, cardiac damage was mainly related to mitochondrial structure and dysfunction, myocardial necrosis and aggravation of apoptosis [23].

We replicated animal models of myocardial injury using selenium-deficient and low protein diets in this study. All different treatment groups have the similar damage in different degrees compared with the doxorubicin group, which is the positive group of myocardial injury. Degeneration and necrosis of the subendocardial myocardium, ventricular septum and parenchyma were observed under a light microscope. Individual myocardial cell pyknosis, deep staining and small focal necrosis were accompanied by an infiltration of inflammatory cells. Myocardial mitochondrial swelling was notable under an electron microscope. Many mitochondria were dissolved, and some myofibril fibers were broken. Myofilament was decomposed, and the sarcoplasmic reticulum was dilated. A contraction band, multiple calcifications around the mitochondria, mitochondrial membrane rupture, lamellar body formation and myeloid changes were observed. These finding are consistent with those obtained in the Yang FY study [24].The above experimental results indicate that the myocardial injury model induced by selenium deficiency and low protein intake was replicated successfully, which lays a foundation for the study of myocardial injury mechanisms.

Selenium deficiency reduces the synthesis of Gsh-px, which in turn reduces the body's antioxidant function. Low nutrition is mainly due to inadequate protein intake which means lack of methionine in the body. Methionine deficiency will lead to hindrance of protein synthesis in vivo, which will lead to excessive oxidation of membrane lipids caused by oxygen free radicals in vivo, damage non-sulfur redox systems, and decrease the activity of glutathione peroxidase. The lack of selenium and protein at the same time can aggravate lipid peroxidation and oxidative changes of low density lipoprotein, thus impairing cardiovascular function. The levels of blood selenium and GSH-PX activity in patients with Keshan disease are decreased. In our study, the concentrations of blood Se and GSH-Px activity were significantly lower in the Se-deficient rats than in the normal controls. The selenium contents and GSH-Px activities in rats fed selenium-deficient or/and low proteindiets were decreased, which was consistent with the results of Zeng XH et al. [25].

The T-AOC oxidative defense system consists of enzymatic and nonenzymatic reactions, reflecting the total antioxidant capacity of the organism [26]. MDA is a lipid peroxidation product formed by the attack of free radicals in biomembrane structures by free radicals. The level of MDA can reflect the degree of cellular lipid peroxidation damage and the presence of reactive oxygen species. ROS are mainly produced by the oxidative phosphorylation of the respiratory chain in the mitochondrial inner membrane. In the body, the stimulation of unfavorable factors can promote the mitochondria to produce ROS, destroy the antioxidant defense system, cause cell structure abnormalities and dysfunction, and induce oxidative stress [27–30]. In this study, the levels of MDA and ROS in the serum and myocardium were significantly higher in the treatment group than in the control group. The levels of T-AOC in the serum and myocardium were significantly lower

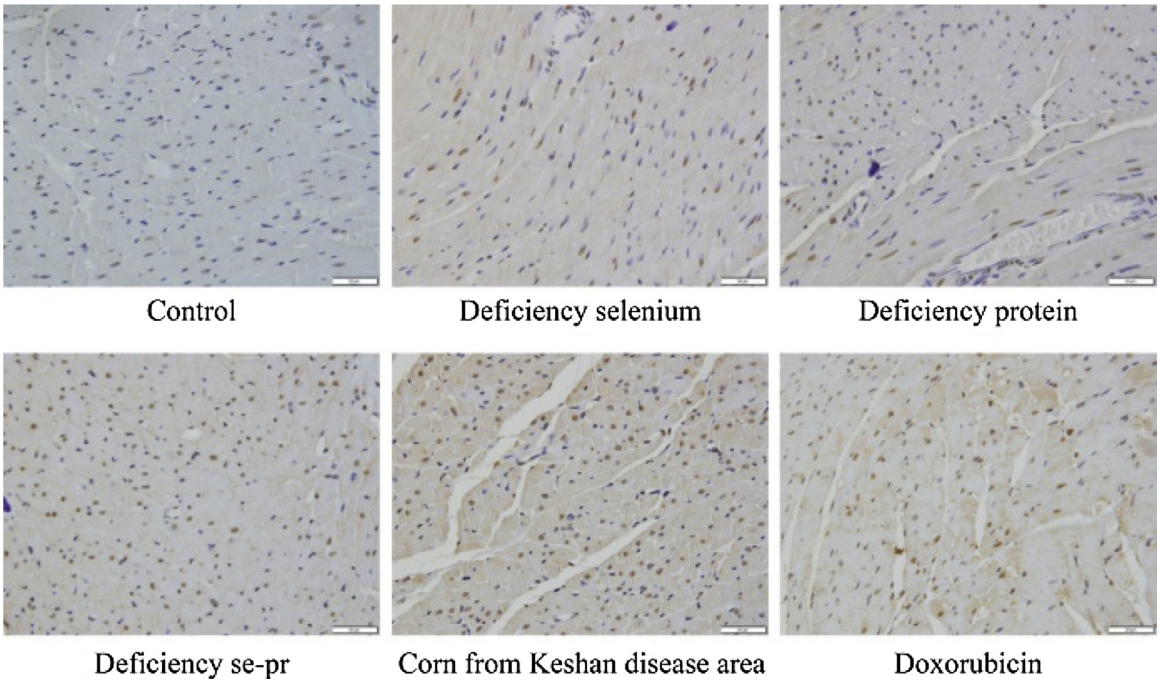

Fig. 12. Relative apoptotic cells in the control and experimental groups (200-fold magnification). Compared with the control group, the apoptotic cells in the experimental group gradually increased. In corn of Keshan disease area group and doxorubicin group had extensive areas of apoptotic cell expression, and the brown-yellow staining were stronger than the other groups. Regardless of the distribution of apoptotic cells or the degree of staining, the change of the protein and selenium-deficient group was more obvious than selenium-deficients group and low-protein group, but less than corn of Keshan disease area group and doxorubicin group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

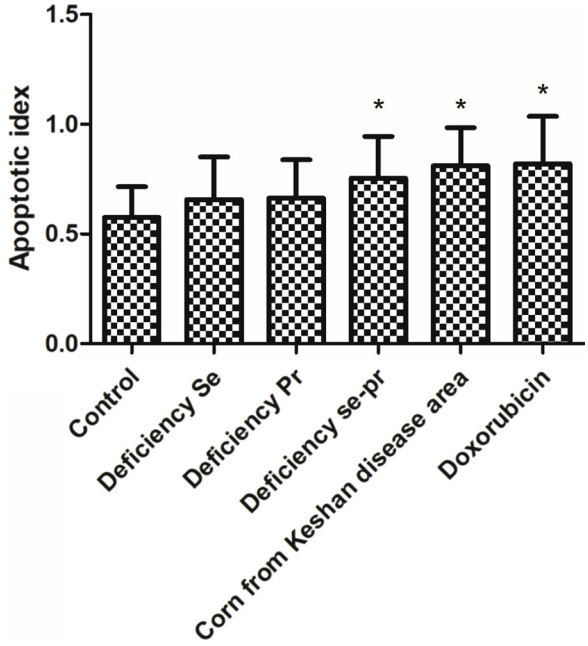

Fig. 13. Relative OD value of apoptotic cell in the myocardial tissue samples in different groups. *P < 0.05 vs. control.

in the treatment group than in the control group. These results indicated that lipid peroxidation in the selenium-deficientand low protein group was enhanced and cell function was affected, which resulted in oxidative damage in the body. This finding is consistent with that of a previous study that found that selenium-deficient rats had decreased antioxidant capacity, excessive free radical production, lipid peroxidation, and cardiac tissue damage [31].

Mitochondria play a decisive role in the apoptotic pathway [32]. The mitochondrial pathway is one of the major mechanisms for apoptosis, and it is a highly conserved process that is regulated by the apoptosis gene. After the apoptosis signals are received by the mitochondria, the permeability of the mitochondrial membrane is changed, which leads to related substances (Protease Caspase、Bcl-2) from the mitochondria entering the cytoplasm and triggers cell apoptosis. Thus, mitochondria may be the master switch in apoptosis. The mitochondrial pathway of apoptosis is regulated by the Caspase family, the Bcl-2 family, Smac and IAP. Normally, Bcl-2 and Caspase are mutually restricted to maintain a balance of cell proliferation and death [33,34], and if this balance is disturbed, apoptosis occurs. In this study, we found that the scattered nuclei were stained with brown in rat myocardial tissue of the selenium-deficient and low-protein groups. The number of apoptotic cells in yellow increased, which was significantly different from the results obtained from the control group. The expression of caspase-9 and caspase-3 was enhanced. The expression of Bcl-2 was weakened, which is crucial in the process of cardiomyocyte apoptosis. In summary, when selenium deficiency and a low protein diet cause myocardial injury in rats, oxidative stress damage occurs in myocardial tissue, resulting in the degradation of mitochondrial membrane potential, matrix edema, rupture of the outer membrane, and activation of caspase-9. The amplifying form activates procaspase-3 into caspase-3, inhibits Bcl-2 expression, leads to apoptosis, enhances mitochondrial apoptosis, and aggravates myocardial damage.

Regardless of body weight gain, pathological changes, redox reaction system indicators and expression of apoptotic regulatory genes, the changes of rats in corn from Keshan disease area group were significantly higher than those in Deficiency selenium and protein group with the same nutritional level. This suggests that besides selenium and protein deficiency, other nutritional factors play a role in myocardial injury, such as vitamin E, which may play a role in the pathogenesis of Keshan disease.

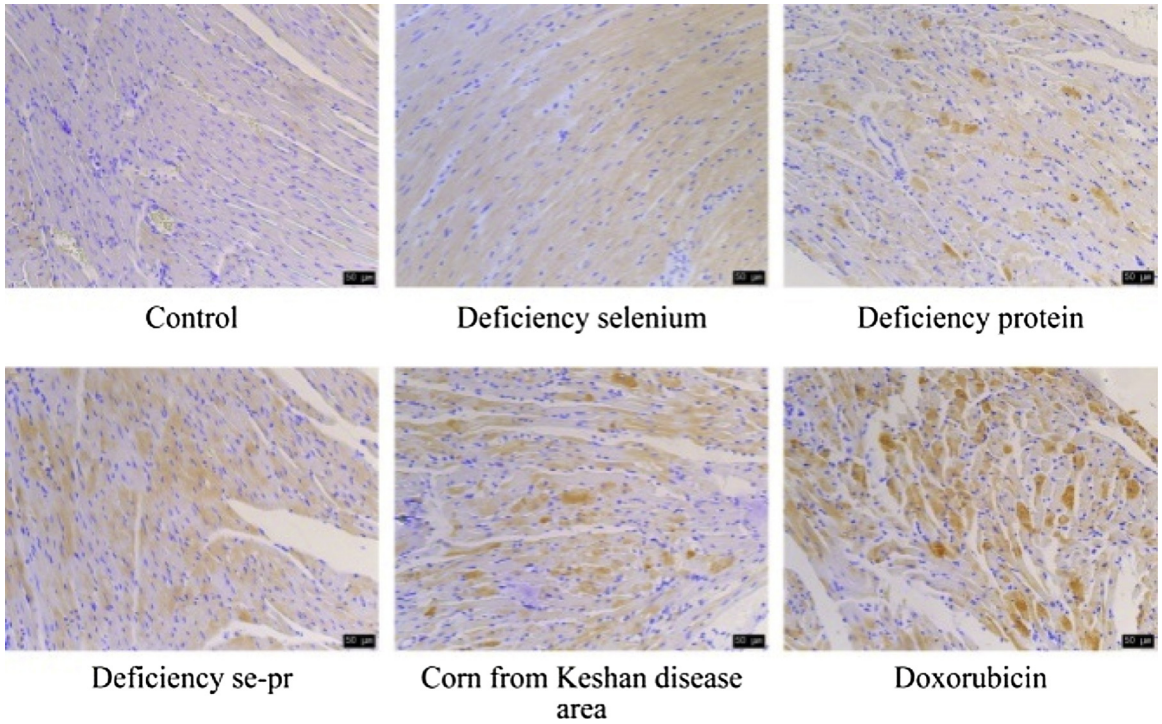

Fig. 14. Relative protein expression of cleaved caspase-9 in the control and experimental groups (400-fold magnification). Compared with the control group, the protein expression of cleaved caspase-9 in the experimental group gradually increased. In corn from Keshan disease area group and doxorubicin group had extensive areas of cleaved caspase-9 protein expression, and the brown-yellow staining were stronger than the other groups. Regardless the range of cleaved caspase-9 protein expression or the degree of staining, the change of the protein and selenium-deficient group was more obvious than selenium-deficient group and low-protein group, but less than corn of Keshan disease area group and doxorubicin group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

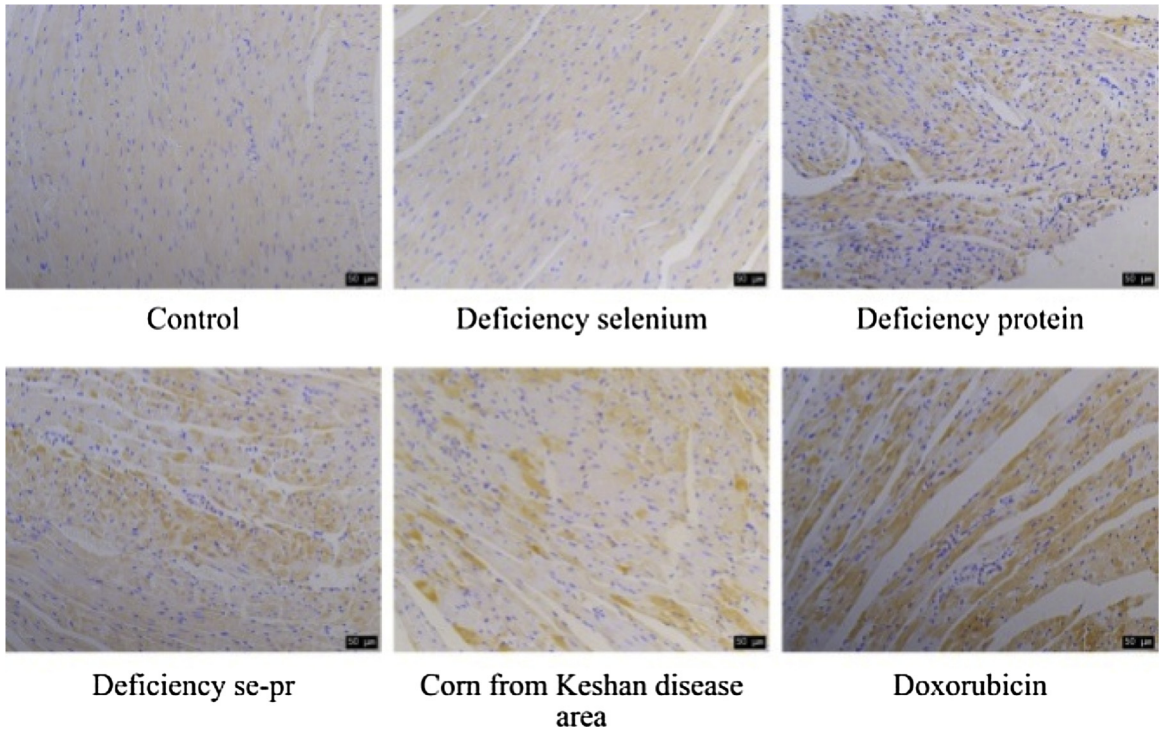

Fig. 15. Relative protein expression of cleaved caspase-3 in the control and experimental groups (400-fold magnification). Compared with the control group, the protein expression of cleaved caspase-3 in the experimental group gradually increased. In corn from Keshan disease area group and doxorubicin group had extensive areas of cleaved-caspase3 protein expression, and the brown-yellow staining were stronger than the other groups. Regardless the range of cleaved caspase-3 protein expression or the degree of staining, the change of the protein and selenium-deficient group was more obvious than selenium-deficient group and low-protein group, but less than corn of Keshan disease area group and doxorubicin group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

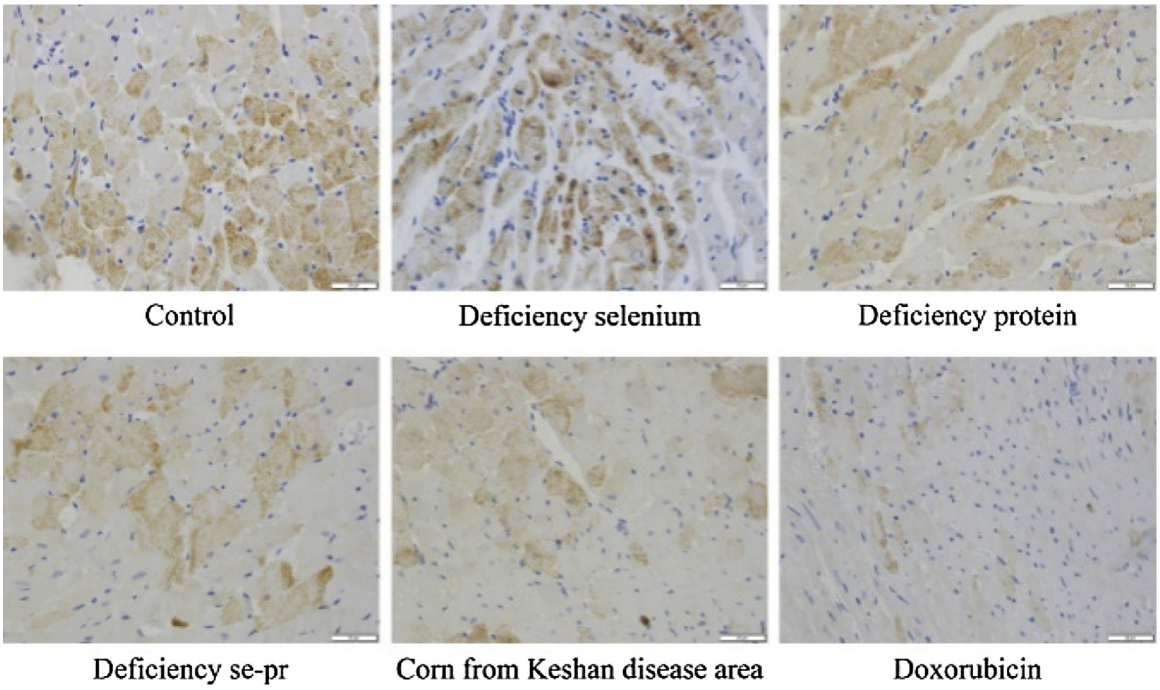

Fig. 16. Relative protein expression of Bcl-2 in the control and experimental groups (400-fold magnification). Compared with the control group, the protein expression of Bcl-2 in the experimental group gradually decreased. The control group has extensive areas of Bcl-2 protein expression, and the brown-yellow staining was stronger than the other groups. According to range size of yellow staining and its strength, the order of protein expression in each experimental group was as follows: selenium-deficient group, low-protein group, protein and selenium-deficient group, corn from Keshan disease area group, and doxorubicin group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

5. Conclusions

In conclusion, our research work suggests that the combination of deficiency selenium and low protein diet is one of the risk factors for Keshan disease. MDA and ROS increase and T-AOC decreases in low selenium/protein diets and Keshan diet, which indicates oxidative damage of cardiomyocytes. Meanwhile, variation of cleaved caspase-9, cleaved caspase-3 and BCL-2 illuminate that cardiomyocyte apoptosis is mediated by mitochondrial pathway. The findings will help us better understand selenium and protein deficiency-related cardiomyopathy and provide possible new therapeutic targets such as the use of selenium and protein supplementation for cardioprotection. However, the mechanisms linking mitochondria-mediated apoptosis related to selenium deficiency and low protein have not yet been clarified. Further studies in this field will be needed to elucidate the interactions between the mitochondria-mediated apoptosis pathway and selenium and protein depletion, which can be integrated into the molecular mechanisms used for the treatment of cardiomyopathy.

Funding

This work was supported by the Department of Education of China (2011TD003), Scientific Research Topics of Heilongjiang Health and Family Planning Commission (2018550), Heilongjiang Provincial Higher Education Fundamental Scientific Research Project (31041180042).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgment

The authors' responsibilities were as follows-DJS: designed the

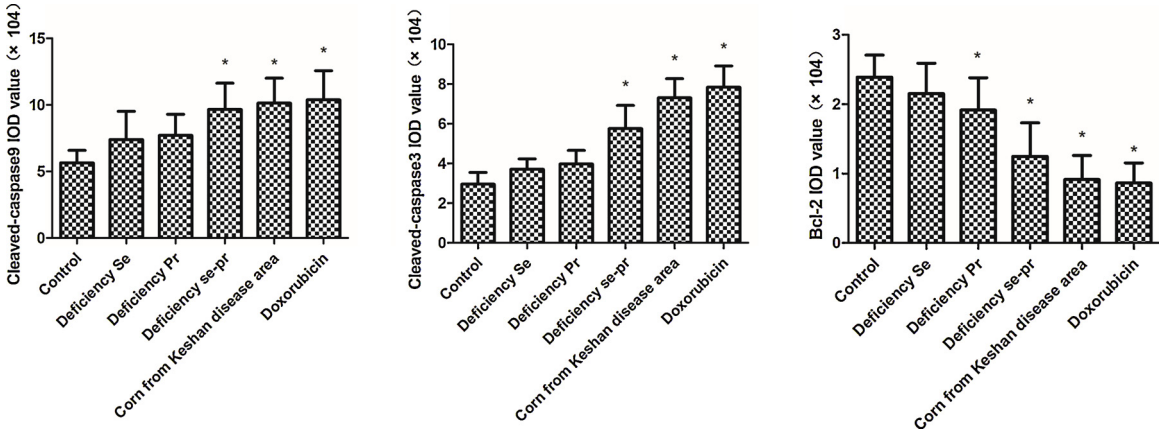

Fig. 17. Relative OD value of cleaved caspase-9、cleaved caspase-3、Bcl-2 protein in the myocardial tissue samples in different groups. *P < 0.05 vs. control.

research, wrote the statistical analysis plan, and had primary responsibility for final content; LWZ, YHG, HQF, NZ and KWW: conducted the research and collected the data; LWZ: analyzed the data; LWZ and DJS: wrote the first draft of the manuscript; LWZ and DJS: interpreted the data; and all authors: contributed to writing and editing the manuscript and read and approved the final manuscript.

References

[1] W.M. Tong, F. Wang, Alterations in rat pancreatic islet betacells induced by Keshan disease pathogenic factors: protective actionof selenium and vitamin E, Metabolism 47 (1998) 415–419 https://www.metabolismjournal.com/article/S0026-0495(98)90052-X/pdf.

[2] W.X. Zhang, W.H. Yu, Keshan disease etiology and experimental cardiomyopathy, Chinese Journal of Endemic Diseases 26 (2006) 469–471.

[3] X.H. Wang, Y.Z. Xiang, F.R. Qu, Study on the relationship between selenium nutrition level in internal and external environment and the incidence of Keshan disease, Chin. J. Endemiol. Prevent. 20 (2005) 351–353.

[4] Y.L. Zhou, J.R. Liu, X.L. Jiang, Study on the relationship between selenium, vitamin E and unsaturated fatty acid and the pathogenesis of Keshan disease, J. Bethun Med. Uni. 27 (2001) 365–368.

[5] B.X. Ti, F.S. Han, W.H. Yu, The protective effect of white matter on the myocardium of rats in the diet of the Keshan disease area, Chin. J. Endemic Dis. 21 (2002) 244–247.

[6] L.L. Zhao, J. Chen, Effects of selenium, protein and vitamin E on the expression of selenoprotein mRNA in rat heart, Chin. J. Endemiol. 31 (2012) 279–281.

[7] L.W. Zhou, J.R. Pei, Experimental study on myocardial injury induced by low selenium and low protein, Chin. J. Endemiol. 26 (2007) 361–363.

[8] H. Zhang, T. Wang, Effects of selenium and protein on myocardial morphology and expression of glutathione peroxidase and mitochondrial thioredoxin reductase in rats, Chin. J. Endemic Dis. 31 (2012) 40–45.

[9] X. Liu, Y.H. Zhu, Low selenium and cardiomyocyte oxidative damage mechanism and expression of apoptosis-related genes, Foreign Medical Medical Geography 32 (2011) 241.

[10] R.A. Sunde, mRNA transcripts as molecular biomarkers in medicine and nutrition, J. Nutr. Biochem. 21 (2010) 665–670 https://www.sciencedirect.com/science/article/pii/S0955286310000069?via%3Dihub.

[11] X.K. Zhong, B. Yu, L.J. Zhang, L.W. Zhou, S.J. Qian, H. Li, et al., Myocardial injury of Keshan disease and apoptosis, Chin. J. Endemiol. 16 (1997) 81–82.

[12] Z. Zhu, M. Kimura, Y. Itokawa, Mineral status in seleniumdeficient rats compared to selenium-sufficient rats fed vitamin-free casein-based or torula yeast-based diet, Biol. Trace Elem. Res. 37 (1993) 219–231 https://link.springer.com/article/10.1007/BF02783797.

[13] P.G. Reeves, Components of the AIN-93 diets as improvements in the AIN-76A diet, J. Nutr. 127 (1997) 838S–841S https://academic.oup.com/jn/article/127/5/838S/4724089.

[14] P.G. Reeves, F.H. Nielsen, G.J. Fahey, AIN-93 purified diets forlaboratory rodents: final report of the American Institute ofNutrition ad hoc writing committee on the reformulation of theAIN-76A rodent diet, J. Nutr. 123 (1993) 1939–1951 https://academic.oup.com/jn/article/123/11/1939/4722783.

[15] GB17019-2010, Keshan Disease Area Control Standard, China Standards Press, Beijing, 2011.

[16] GB17020-2010 Keshan Disease Area Judgment and Type Division, China Standard Press, Beijing, 2011.

[17] F.Y. Yang, Keshan disease and myocardial mitochondrial disease, Life Sci. 36 (2006) 481–485.

[18] C. Yan, W. Fang, X.P. Li, W. Cai, Status and etiology progress of keshan disease, Adv. Cardiovasc. Dis. 38 (2017) 225–229.

[19] F. Qiu, Myocardial mitochondrial damage in Keshan disease, Shanxi Med. J. 21 (1992) 159–161 F.

[20] H.Y. Zhang, N. Guo, J. Jiang, D. Yao, Five trace elements in patients with Keshan disease and dilated cardiomyopathy Blood level comparison, Chin. J. Ctrl. Endem. Dis. 31 (2016) 404–406.

[21] N.M. AL-Rasheed, H.A. Attia, R.A. Mohamed, Preventive effects of selenium yeast,chromium pieolinate, zinc sulfate and their conbination on oxidative stress,inflammation,impaired angiogenesis and atherogenesis in myocardial infarction in rats, J. Pharm. Pharm. Sci. 16 (2013) 848–867 https://www.ncbi.nlm.nih.gov/pubmed/24393559.

[22] Y.H. Chan, C.W. Siu, K.H. Yiu, Adverse systemic arterial function in patients with selenium deficiency, J. Nutr. Health Aging 16 (2012) 85–88 PDF http://view.pubtsg.com:8080/enpdf/1964/1964-1-1/Adverse%20systemic%20arterial%20function%20in%20patients[PMIDS22238006].

[23] Y.C. Zhang, Y.H. Dang, H. Wang, Relationship between low selenium and lipid peroxidation and myocardial cell injury in rat myocardial tissue, Heart magazine 15 (2004) 532–534.

[24] F.Y. Yang, Z.H. Lin, J.R. Xing, The relationship between the function and ultrastructure of low selenium and myocardial mitochondrial membrane, Chin. J. Endemic Dis. 11 (1992) 263–266.

[25] X.Y. Zeng, B.C. Zhou, Protein deficiency in myocardial damage under low selenium and high manganese conditions, Chin. J. Endemic Dis. 15 (1996) 330–333.

[26] L. Li, S.M. Huang, S.L. Zhao, The effect of rosmarinic acid on oxidative stress in rats with anti-Thy1.1 mesangial proliferative glomerulonephritis model, Practical Pediatric Clinical J. 22 (2007) 1174–1175.

[27] T.L. Merry, M. Ristow, Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training, J. Physiol-London 594 (2016) 5135–5147 https://physoc.onlinelibrary.wiley.com/doi/full/10.1113/JP270654.

[28] A. Dyson, F. Dal-Pizzol, G. Sabbatini, A.B. Lach, F. Galfo, Dos Santos Cardoso J, etal. Ammonium tetrathiomolybdate following ischemia/reperfusion injury: chemistry, pharmacology, and impact of a new class of sulfide donor in preclinical injury models, PLo S Med 14 (2017) e1002310https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5497958/.

[29] B. Niemann, S. Rohrbach, M.R. Miller, D.E. Newby, V. Fuster, J.C. Kovacic, Oxidative stress and cardiovascular risk: obesity, diabetes, smoking, and pollution: part 3 of a 3-part series, J. Am. Coll. Cardiol. 70 (2017) 230–251 https://www.sciencedirect.com/science/article/pii/S0735109717375101?via%3Dihub.

[30] Y. Wang, Y. Wu, Y. Wang, A. Fu, L. Gong, W. Li, Bacillus amyloliquefaciens SC06 alleviates the oxidative stress of IPEC-1 via modulating Nrf2/Keap1 signaling pathway and decreasing ROS production, Appl. Microbiol. Biotechnol. 101 (2017) 3015–3026 https://link.springer.com/article/10.1007%2Fs00253-016-8032-4.

[31] Y.Z. Wang, H. Bi, J.Y. Zhao, Observation on the protective effect of selenium on myocardial mitochondrial membrane in rats, Trace elements and health research 15 (1998) 5–6.

[32] E.C. Kühn, A. Slagman, E.C. Kühn-Heid, J. Seelig, L. Schomburg, Circulating levels of selenium-binding protein 1 (SELENBP1) are associated with risk for major adverse cardiac events and death, J. Trace Elem. Med. Biol. 52 (2019) 247–253 https://www.sciencedirect.com/science/article/pii/S0946672X18306291?via%3Dihub.

[33] Eric N. Shiozaki, Jijie Chai, Daniel JRigotti, Mechanism of XIAP-mediated inhibition of caspase-9, Mol. Cell 11 (2003) 519–527 https://www.sciencedirect.com/science/article/pii/S1097276503000546.

[34] G.B. Zhou, G.S. Zhang, Indomethacin induces changes in caspase and intracellular free calcium concentration during apoptosis of K562 cells, Chin. J. Hematol. 22 (2001) 241–243.