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Original Article

Aging Induces Heart Injury via Mitochondrial Apoptotic Pathway and Dysregulation of Sirt1/Nrf2 Axis in Male Rats: Rescue Effect of Prazosin

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Abstract

Introduction: Myocardial aging is a significant contributor to heart dysfunction and subsequently heart failure. However, the underlying molecular mechanisms are not well understood. The goal of this study is to investigate how aging and Prazosin treatment affect apoptosis and Sirt1/Nrf2 signaling pathway in the hearts of aging rats.

Methods: Eighteen male Wistar rats including 6 young rats (3-month-old; 200-250 g) in the Young control group and 12 aged rats (18-month-old; 400-450 g) were selected. They were divided into three groups (n = 6): Young control, Aged, Aged + Prazosin (1 mg/kg; ip; intraperitoneally). Apoptosis was examined by TUNEL staining. The expression of Sirt1, Nrf2, Bax, and Bcl2 proteins were investigated by western blotting. Cytochrome c concentration was quantified by ELISA with a commercially available kit. The concentrations of CK-MB and LDH were determined using a spectrophotometric method with an autoanalyzer.

Results: At the end of the experiment, the TUNEL staining indicated that aging increased the number of apoptotic cells in the hearts of male rats. Molecular analysis demonstrated that aging resulted in increased protein levels of cytochrome c, Bax/Bcl-2 ratio and also decreased protein levels of Sirt1 and Nrf2, with a concomitant up-regulation of LDH and CK-MB enzyme activities in the heart tissue compared to the young control group. Sirt1 and Nrf2 were negatively correlated with cytochrome c ($r = -0.74$, $r = -0.90$, $P < 0.001$) and apoptosis ($r = -0.81$, $r = -0.86$, $P < 0.001$).

Conclusion: Our findings indicate that aging damages heart tissue via mitochondrial apoptosis and Sirt1/Nrf2 dysregulation, while prazosin reverses these changes, highlighting its potential as a therapy against age-related cardiac apoptosis. Furthermore, a negative correlation was identified between apoptosis and Sirt1/Nrf2.

Introduction

As aging progresses, the body undergoes various physiological changes that increase susceptibility to numerous chronic diseases, particularly cardiovascular disorders.[1] Aging leads to structural and functional changes in the cardiovascular system, including reduced diastolic function, left ventricular wall thickening, arterial stiffening, and endothelial dysfunction, which collectively increase the risk of cardiovascular complications.[2,3] Aging is closely associated with heightened oxidative stress, a condition caused by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms.[4] This imbalance is a major contributor to cellular damage, driving the aging process and leading to the activation of apoptosis, a regulated form of cell death.[5] While apoptosis is essential for normal cellular turnover, it can exacerbate cardiac dysfunction and contribute to the development of heart failure, a major health concern among the elderly population.[6,7] With aging, an imbalance between pro-apoptotic and anti-apoptotic proteins, such as Bax and Bcl-2, further promotes cell death.[8,9]

Critical regulatory proteins like Sirtuin 1 (Sirt1) and Nrf2 play pivotal roles in protecting against oxidative damage and maintaining cellular homeostasis. These proteins show diminished activity in aged individuals, exacerbating oxidative stress and contributing to cardiac damage. [10-12] Sirt1, a member of the NAD-dependent deacetylase family, is recognized as a significant regulator in a variety of cardiovascular diseases.[13-15] Sirt1 plays a critical role in maintaining mitochondrial function, reducing oxidative damage, mitigating inflammatory responses, and inhibiting apoptosis. [13] Additionally, Sirt1

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has the capacity to regulate key transcription factors such as nuclear erythroid factor 2-related factor 2 (Nrf2), which is essential for cytoprotection, the anti-inflammatory response and the antioxidant defense.[14] Numerous studies have explored the significant role of the Sirt1/Nrf2 pathway in various age-related conditions, including renal aging [16], hypertension-induced renal damage [17], age-related decline in ovarian function [18], neurodegenerative disorders such as Alzheimer's disease [19], cognitive impairment [20], osteoporosis and bone mass regulation [21], liver aging [22], and thyroid dysfunction.[23] These findings emphasize how Sirt1/Nrf2 signaling reduces oxidative stress, maintains cellular function, and slows down aging processes. Nevertheless, thus far, only a few studies have described the potential role of Nrf2 and its non-pharmacological induction in cardiac aging.[24,25] However, there are not enough studies in context of Sirt1/Nrf2 signaling pathway in the hearts of aging rats. In addition, the role of Nrf2 has been established in d-galactose accelerated aging model.[11,26] Considering the above, the role of the Sirt1/Nrf2 pathway in the naturally aging heart has not been explored. Herein, we decided in this study to evaluate Sirt1/Nrf2 pathway in naturally aged heart.

Prazosin, a selective α1-adrenergic receptor antagonist commonly used for managing hypertension, has garnered attention as a potential therapeutic agent for protecting cardiac tissue from the harmful effects of oxidative stress and apoptosis in various normal, benign, and malignant cells.[27] Prazosin is capable of crossing the blood-brain barrier, and studies have demonstrated that its peripheral administration can modulate adrenergic activity within the central nervous system.[28] Moreover, prazosin is used in treating several kinds of cancer [29-31], ischemic damage [32], Alzheimer's disease [33] and cognitive dysfunction in aged animals.[34] Although prazosin has shown promising effects in various contexts, its influence on apoptosis and its mechanisms in the aging heart has not been fully explored. Therefore, this study seeks to examine how aging affects the Sirt1/Nrf2 signaling pathway in cardiac tissues and to explore the potential protective benefits of prazosin in aged male rats. By uncovering the molecular mechanisms behind prazosin's actions, this study aims to contribute valuable insights that could lead to the development of novel therapies for age-related heart dysfunction.

Materials and Methods

Animals and Study Design

Eighteen male Wistar rats were obtained from the animal house facility of Urmia University of Medical Sciences. All rats were maintained under standard conditions (22 ± 3°C, 55 ± 5% humidity, and a 12-hour light/dark cycle) with food and water available ad libitum. After a one-week acclimatization period, the rats were randomly divided into three groups (n = 6 per group): 1) Young control group (3-month-old rats): Young rats received normal saline for one month. 2) Aged group (18-month-old rats): Aged rats received normal saline for one month. 3) Aged + prazosin group: Aged rats received intraperitoneal prazosin (1 mg/kg) [27,32] dissolved in normal saline daily for 30 days.

All procedures involving animals were conducted in compliance with the Institutional Animal Ethics Committee guidelines of the Urmia University of Medical Sciences (IR.UMSU.AEC.1401.029).

Tissue Collection and Preparation

At the end of the treatment period, the rats were anesthetized with intraperitoneal injection Ketamine (80 mg/kg) and xylazine (10 mg/kg). The thoracic cavity was opened, and the hearts were rapidly excised. Excess fats were removed, and the tissues were rinsed in cold phosphate-buffered saline (PBS). The Apex of the heart tissue was fixed in 10% formalin for histological analysis, while a portion of the left ventricle tissue was used for biochemical assays. For biochemical analysis, heart samples were homogenized (10% w/v) in phosphate buffer (pH 7.4), centrifuged, and the supernatant was collected and stored at -80°C until further analysis.

Apoptosis Analysis Using TUNEL Assay

Apoptosis in hippocampus tissue was detected using the In Situ Cell Death Detection Kit (Roche Molecular Chemicals, cat. no. 11684817910) according to kit instructor. Briefly, 5 µm-thick sections from formalin-fixed, paraffin-embedded hippocampus tissue were deparaffinized by immersing them in xylene, rehydrated, and washed in PBS. Then, by using proteinase K (20 µg/ml), the sections were permeabilized and were washed in PBS. To inhibit endogenous peroxidase activity, the sections were exposed to 3% hydrogen peroxide. Subsequently, 50 µL of TUNEL solution was added to each section and incubated at 37 °C for one hour. Finally, the results were obtained under a fluorescence microscope (Olympus BX50). The nuclei of total cells were stained blue by DAPI and the apoptotic cells were labeled bright green by TUNEL. For assessing the apoptotic cells, four non-overlapping fields of view per section from two-to-three sections (from different regions of the hearts) per animal were analyzed. For each field of view, the number of positively apoptotic cells (labeled bright green by TUNEL) and the total number of cells (stained blue by DAPI) were counted as mean ± S.E.M in a blinded manner by two independent investigators. Apoptotic index in each group was expressed by the ratio of apoptotic cells to DAPI-positive cells (magnification × 400).

Western Blot Analysis

Western blotting was performed to evaluate the expression levels of Sirt1, Nrf2, Bax, and Bcl2 proteins. Heart tissues were homogenized in a lysis buffer containing EDTA (0.003 g), NaCl (0.08 g), sodium deoxycholate (0.025 g), SDS (0.01 g), protease inhibitor cocktail, and NP40 (1%) Triton (10 µL). The lysates were centrifuged at 12,000 rpm for 10 min, and the supernatant was collected for protein quantification. Equal amounts of protein samples were separated using SDS-PAGE (10%) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and 0.1%

Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature to prevent nonspecific binding. Primary antibodies against Sirt1, Nrf2, Bax, and Bcl2 were incubated overnight at 4°C, followed by incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h (Table 1). β-actin was used as the internal control. The immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) system, and the relative expression levels were quantified using ImageJ software.

ELISA Method

For estimation cytochrome c level, the supernatant sample was used by the quantitative sandwich enzyme immunoassay method using a related ELISA kit (China; Cat. NO. CSB-EL006328RA) in accordance to the manufacturers' instructions.[35]

Determination of CK-MB and LDH Activities

The concentrations of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were determined using a spectrophotometric method with an autoanalyzer (Roche Cobas-Integra 800) and commercially available test cassettes (Roche). Additionally, CK-MB levels were evaluated spectrophotometrically via an immunoinhibition technique using the same autoanalyzer (Cobas Integra 800).

Statistical Analysis

Statistical analyses were performed using SPSS software version 23. Data normality was assessed using the Shapiro-Wilk test, followed by one-way ANOVA and Tukey's post hoc test for multiple group comparisons. Pearson's correlation test was used to assess the correlation between apoptosis and Sirt1/Nrf2. Results are expressed as mean ± SEM, and a p-value < 0.05 was considered statistically significant.

Results

Apoptotic cells in the heart tissue

The apoptotic cells of heart tissue were evaluated using a TUNEL method. Accordingly, the apoptotic index in the aged group was significantly higher ($P < 0.001$) than the young rats. Interestingly, prazosin treatment significantly reduced the number of apoptotic index in aged rats ($P < 0.001$) (Figure 1 a, b).

Nrf2, Sirt1 levels and Bax/Bcl-2 Ratio in the heart tissue

The protein levels of Sirt1, Nrf2, Bax and Bcl-2 were measured by western blot analysis. In our study, both Nrf2 and Sirt1 expression in the cardiac tissue of aged rats was significantly reduced compared to young rats ($P < 0.001$). However, prazosin treatment significantly enhanced the levels of these proteins in the heart tissue of aged rats ($P < 0.001$ for Nrf2, $P < 0.05$ for Sirt1) (Figure 2 a, b, c).

Then we conducted a further investigation into the impact of prazosin on the intrinsic apoptotic pathway, namely the mitochondrial apoptotic pathway in aged rats, which is known to play a crucial role in regulating apoptosis. Bax is a pro-apoptotic protein, and its upregulation shifts the balance toward apoptotic signaling. In contrast, Bcl-2 is a key anti-apoptotic protein that functions to inhibit apoptosis. In this study, aging increased the Bax protein expression ($P < 0.001$) and decreased Bcl-2 protein expression ($P < 0.01$). So, Bax/Bcl-2 ratio was significantly elevated in the cardiac tissue of aged rats compared to young rats ($P < 0.001$). Notably, prazosin treatment markedly reduced Bax/Bcl-2 ratio in the heart tissue of aged rats ($P < 0.001$) (Figure 2 a, d).

Cytochrome c level in the Heart tissue

Cytochrome c is a pro-apoptotic molecule released from the mitochondria that triggers the apoptotic process. Figure 3 demonstrated that, cytochrome c levels were significantly higher in the cardiac tissue of aged rats compared to young rats ($P < 0.001$), while prazosin treatment significantly reduced these levels in aged rats ($P < 0.05$).

Serum LDH and CK-MB Levels

LDH and CK-MB levels were measured as indicators of heart tissue injury. The aged rats exhibited significantly higher levels of LDH and CK-MB compared to the young rats ($P < 0.001$). However, prazosin therapy significantly reduced these elevated levels for LDH ($P < 0.001$) and CK-MB ($P < 0.01$) (Table 2).

The correlation among sirt1, Nrf2, cytochrome c and apoptotic index

We assessed the correlation among sirt1, Nrf2, cytochrome c and apoptotic index in the heart tissue of rats. As shown in Figure 4, Sirt1 and Nrf2 is negatively correlated with cytochrome c ($r = -0.74$, $r = 0.90$, $P < 0.001$) and apoptosis ($r = -0.81$, $r = -0.86$, $P < 0.001$).

Discussion

In this study, we found significant age-related changes in cardiac tissue, indicating an increase in oxidative stress as demonstrated in our previous study [36], which is manifested by an increase in MDA and a decrease in SOD levels (data not shown). The Apoptotic index, as a marker of cell death, was increased in the hearts of aged rats as evidenced by upregulation of Bax/Bcl-2 ratio and cytochrome c level, pointing to the stimulation of mitochondrial apoptotic pathway. Further molecular analysis showed that Nrf2 and Sirt1 protein expression was notably reduced in aging rats. Interestingly, remarkable amelioration of the molecular alterations in the heart tissue and, subsequently, the reduction of apoptotic cells to those observed in the aged animals were exhibited in the prazosin-treated rats.

Aging is a major contributor to cardiovascular diseases (CVDs), primarily due to accumulated oxidative stress and mitochondrial dysfunction.[37,38] This gradual damage releases LDH and CK-MB into circulation reflecting cumulative cellular membrane disruption, and impaired metabolic hemeostasis.[39] Increased levels of these enzymes can serve as a biomarker for damaged heart tissue which is observed in our study. Disruptions in mitochondrial

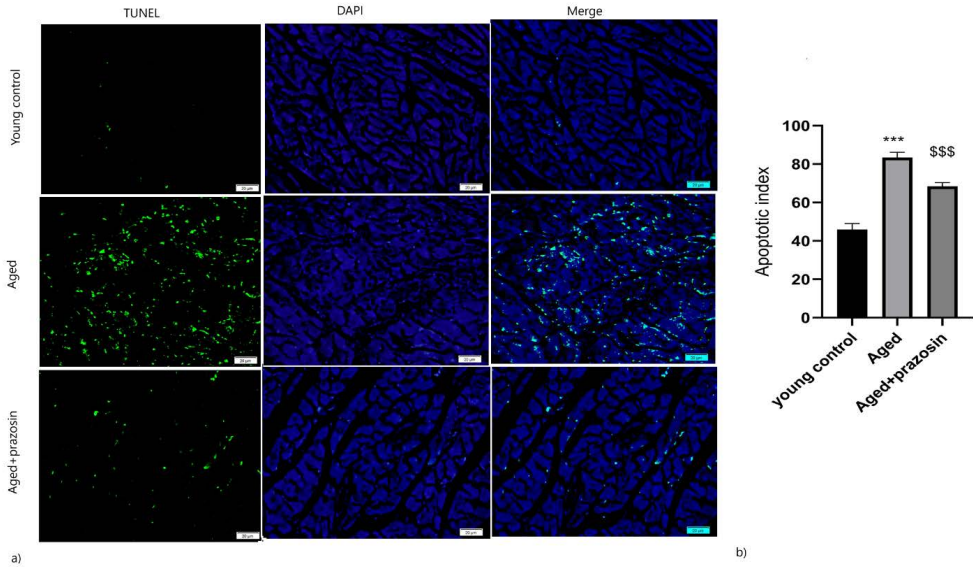


Figure 1. Apoptosis index in the heart tissues of different groups. a) Images of apoptotic cells in the heart tissue of male rats in different groups with TUNEL staining. Magnification × 400. b) Quantitative analysis of the apoptotic index (percentage of TUNEL-positive nuclei, %). All data are represented as the mean ± SEM (n = 6). *** $P < 0.001$ compared with young control group. $$$ $P < 0.001$ compared with aging group. Scale bars are as indicated.

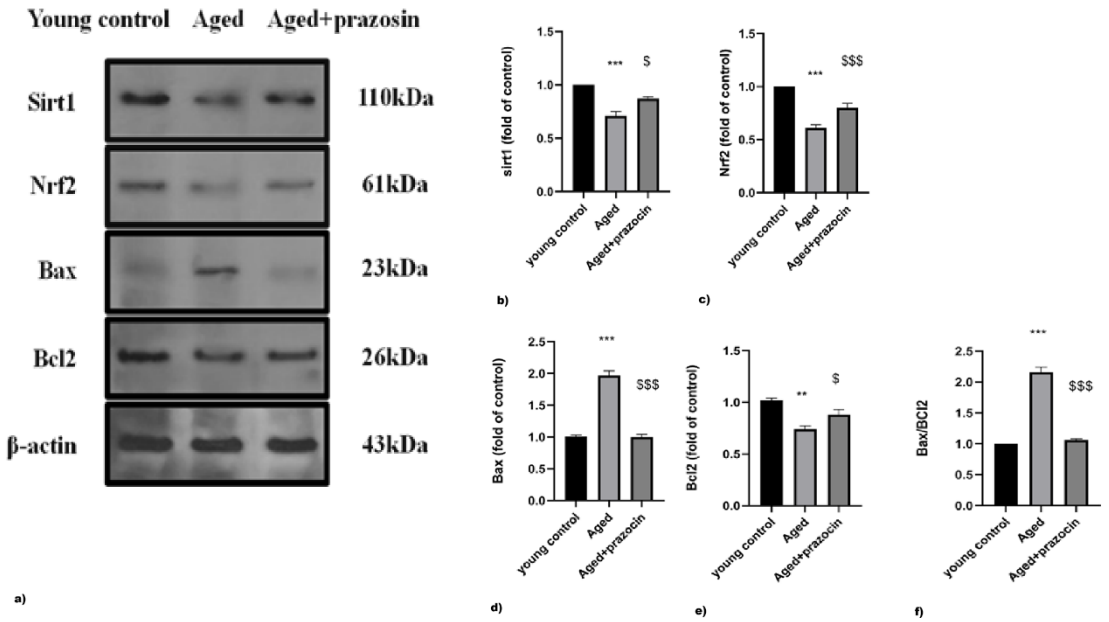


Figure 2. Sirt1, Nrf2, Bax, Bcl2protein expressions and Bax/Bcl2 ratio in the heart tissues of different groups a) The blotting images of c Sirt1, Nrf2, Bax, Bcl2 and Bax/Bcl2 ratio b, c) The bar charts represent the quantitative analysis of Sirt1, Nrf2, Bax, Bcl2 and Bax/Bcl2 normalized against β-actin. All data are expressed as the means ± SEM (n = 6). ** $P < 0.01$, *** $P < 0.001$ compared with young control group. $$P < 0.05$, $$$P < 0.001$ compared with aging group.

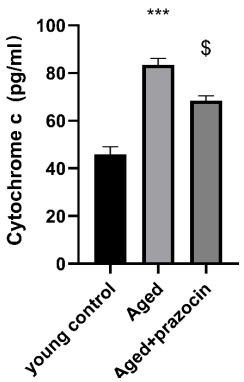


Figure 3. cytochrome c level in the heart tissues of different groups. All data are expressed as the means ± SEM (n = 6). *** $P < 0.001$ compared with young control group. $$P < 0.05$, compared with aging group.

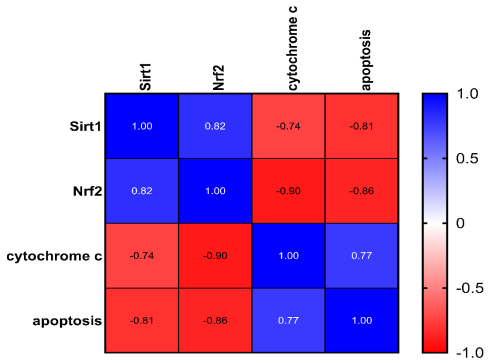


Figure 4. The relationship among sirt1, Nrf2, cytochrome c and apoptotic index. Sirt1 and Nrf2 levels are negatively correlated with cytochrome c (r = -0.74, r = 0.90, $P < 0.001$) and apoptosis (r = -0.81, r = -0.86, $P < 0.001$)

Table 1. The antibodies used in Western blotting assays.

Primary antibody	Company	Dilution	Catalog number
Sirt1	ABCAM	1:300	ab189494
Nrf2	SANTA CRUZ	1:300	sc-365949
Bax	SANTA CRUZ	1:300	sc-7480
Bcl-2	SANTA CRUZ	1:300	sc-492
β-Actin	SANTA CRUZ	1:300	sc-47778

homeostasis lead to excessive ROS production, which in turn triggers cytochrome c release and apoptosis.[40-44] Cytochrome c is a key mitochondrial protein involved in both energy production and apoptosis. During aging, its release into the cytoplasm triggers caspase activation, leading to increased cell death.[45] Rizvi et al. linked increased ROS to oxidative mitochondrial damage and cytochrome c release in aging hearts.[46] The resulting imbalance in apoptotic regulators, such as an increased Bax/Bcl-2 ratio and cytochrome c level promotes cardiac dysfunction and death [44,47,48] which are in agreement with our study. However, the molecular mechanism of apoptosis in aging heart was not clearly defined.

There is increasing evidence to declare Sirt1 involvement as a key regulator in inflammation, apoptosis and antioxidant defense systems by activating Nrf2 pathway to reduce ROS production.[49] In addition, Sirt1 and Nrf2 serve as pivotal modulators of apoptosis in various pathological conditions.[13,50] Sirt1, a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, plays a key role in apoptosis regulation by deacetylating proteins such as p53 and Bcl-2 family members.[49] Moreover, Sirt1 exerts cytoprotective effects by interacting with Nrf2, a major regulator of antioxidant defense mechanisms.[51] Our results in the present study clarified that Sirt1 and Nrf2 expression levels were significantly lower in the cardiac tissue of aged rats compared to young ones. This aligns with the study by Alcendor et al, demonstrating that Sirt1 plays a crucial role in cardiac aging by reducing fibrosis, apoptosis, and oxidative stress.[52] Similarly, Luo et al. found that moderate activation of Sirt1 can help slow down cardiovascular aging, but excessive Sirt1 overexpression may lead to increased oxidative stress and cardiac dysfunction.[53] In addition, Yang et al. explored how Nrf2 activation alleviated oxidative stress and enhanced antioxidant responses in aging induced by D-galactose. [11] Collectively, these findings highlight the critical role of the Sirt1/Nrf2 axis in apoptosis regulation across various pathological contexts. By modulating mitochondrial function and apoptotic pathways, Sirt1 and Nrf2 contribute significantly to cellular survival and homeostasis. This is the first study to identify that natural aging causes heart apoptosis and Sirt1/Nrf2 alteration. These results underscore the therapeutic potential of targeting Sirt1/Nrf2 signaling to mitigate age-related cardiac dysfunction. In addition, Sirt1 and Nrf2 in the heart of aged rats was negatively correlated with cytochrome c and apoptosis which partly reflected heightened heart apoptosis through Sirt1/Nrf2 pathway. Future studies focusing on pharmacological interventions targeting this pathway may provide promising therapeutic strategies for conditions characterized by excessive oxidative stress and apoptosis, such as myocardial ischemia.

Table 2. The levels of LDH and CKMB in different groups.

Groups	Young control	Aged	Aged+prazosin
LDH (U/L)	1209.5 ± 132.84	2791.7 ± 171.28***	1768.3 ± 265.89 $$$
CK-MB (U/L)	109.33 ± 11.94	295 ± 23.83***	251.67 ± 22.93 $$

***p < 0.001 vs young control rats, $$ p < 0.01, $$$ p < 0.001 vs aged group. All data are expressed as the means ± SEM (n = 6).

Since aging is closely associated with an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms namely oxidative stress, it is necessary to combat with this hazardous effect by using antioxidant strategies.[54] The second point addressed in this study was that prazosin treatment as an antioxidant agent, attenuated aging heart apoptosis, which was evidenced by upregulation of Bax/Bcl-2 ratio and cytochrome c level. This finding is in agreement with the study of Malekinejad et al, in which administration of prazosin exerted an anti-apoptotic effect by increasing the bcl-2 levels and decreasing the expression of Bax and caspase 3 enzymes in heart damage induced by renal ischemia in rats.[27] Moreover prazosin has been observed to modulate the apoptotic pathways in endothelial progenitor cells (EPCs), and protect against ischemic damage in cerebral infarction.[32] In another study, it was indicated that prazosin treatment reduced apoptosis of myocardial tissue through regulation of ERK pathway.[55] At the molecular level, Prazosin effectively restored Sirt1 and Nrf2 proteins, which suggests that the protective effect of prazosin on heart tissue in aging rats might result, in part, from a reduction of apoptotic index and regulating Sirt1/Nrf2 axis. Based on the literature, Prazosin has antioxidant capacities as evidenced by increasing the level of GSH as a scavenger of free radicals and the activity of CAT enzyme [27], also upregulates SOD and HO-1 activation and downregulates oxidative stress status.[55] In this study, Prazosin could restore Sirt1 and Nrf2 proteins in aging heart, considering that this effect is due to its antioxidant effect by reducing ROS production and upregulation of antioxidant enzymes. Based on above information, prazosin has a secondary or indirect effect on this pathway.

However, there are some limitations in our study, one is the use of only male rats and thus potential sex-specific differences particularly those related to hormonal regulation, were not addressed. Female hearts are influenced by fluctuating sex hormones, especially estrogen, which has been shown to modulate oxidative stress, inflammatory responses, apoptotic pathways, and adrenergic receptor signaling. The other limitations include not measuring hemodynamic parameters, not applying molecular inhibitors and not assessment of downstream molecules to validate the pathway involved in this hazardous effect of aging heart and the protective effect of prazosin. Also, the effect of prazosin administration was checked after 30 days of treatment and whether this protection continues at later time points remains elusive.

Conclusion

Overall, our data describe that the pathogenesis of heart aging is linked to apoptosis partly mediated by Sirt1/Nrf2 axis. Sirt1/Nrf2 is negatively correlated with apoptosis indicating that Sirt1/Nrf2 pathway may play a potential role in the heart apoptosis in aged rats. However, prazosin treatment could alleviate the heart apoptosis as manifested by Bax, Bcl-2 and cytochrome c alterations. Of note, prazosin can exert this effect probably by modulating Sirt1/Nrf2 axis and therefore favoring heart survival in the context of aging. Further research is warranted to explore its long-term cardioprotective effects and potential clinical applications.

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Authors' Contribution

Conceptualization: Nasrin Mehran Fard, Roya Naderi
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Supervision: Roya Naderi
Writing–original draft: Aileen Saranjam, Roya Naderi
Writing–review & editing: Roya Naderi

Clinical trial number

Not applicable.

Competing Interests

The authors declare no conflict of interest.

Ethical Approval

All procedures involving animals were conducted in compliance with the Institutional Animal Ethics Committee guidelines of the Urmia University of Medical Science (IR.UMSU.AEC.1401.029).

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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