Original Article

Eplerenone Reverses Age-Dependent Cardiac Fibrosis by Downregulating Osteopontin

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ABSTRACT

Purpose: The risk of cardiovascular disease increases dramatically with aging. It is now fully understood that fibrotic remodeling is the primary cause of cardiac structural and functional changes related to the normal aging process. However, the related signaling pathways and mechanisms are not completely understood. Therefore, finding new therapeutic approaches targeting cardiac fibrotic remodeling may be necessary to develop preventive care in the geriatric population against cardiovascular diseases. In this study, we evaluated the potential role of osteopontin (OPN) as a novel mediator of age-dependent fibrotic pathways in the heart, as well as the effect of eplerenone on cardiac fibrosis reversal.

Methods: Fischer-344 (F-344) rats were used in 3 groups: young rats (2–3 months old), aged rats (22-24 months old) without any treatment, and aged rats treated with eplerenone (100 mg/kg/day) for 2 weeks. The mRNA expression level of OPN was evaluated using real-time PCR, and histological assessments were done to assess cardiac tissue fibrosis.

Results: The expression level of OPN was significantly higher in aged rats than in young rats. Treatment with eplerenone significantly attenuated the level of OPN and cardiac fibrosis compared with untreated aged rats.

Conclusions: Targeting cardiac fibroblast function with eplerenone could decrease the expression of the OPN marker and reverse age-related cardiac fibrotic changes. ***(Iranian Heart Journal 2024; 25(2): 6-14)***

KEYWORDS: Cardiac remodeling, Aging, Fibrosis, Osteopontin, Eplerenone, Inflammation

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The prevalence of atrial fibrillation (AF) is increasing worldwide, with high morbidity, mortality, and health care costs. [1] The aging of the general population is the main cause of the growing incidence and prevalence of AF. [2, 3] Since age is an unmodifiable risk factor, the probability of the onset of new AF doubles with each decade of age. The prevalence of AF is 0.12–0.16% in young people aged ≤49 years, 3.7–4.2% in the population between 60–70 years old, and 10–17% in older people aged ≥80 years. [4] Consequently, the risk of AF with aging is very common even in healthy subjects without a history of cardiovascular diseases or risk factors for AF. [5]

In this regard, considering aging in cardiac remodeling studies is critical to providing new insights for preventing arrhythmogenic atrial remodeling and introducing novel therapies for AF. [6] Unfortunately, basic and clinical research on cardiac structural and functional changes related to the normal aging trend has been limited. Fibroblast activation is one of the main causes of arrhythmogenic cardiac remodeling, ultimately interrupting the coupling between cardiomyocytes and electrical signal propagation. [7, 8] Moreover, age-related fibrotic patterns and the quality of the collagen network may impact myocardial stiffness or signal propagation.

Although it is known that reactive fibrosis increases left ventricle stiffness, [9] electrical mapping studies suggest that the architecture of fibrotic remodeling in the heart differentially impacts electrical propagation and conduction delay. [10] In this regard, considering aging in cardiac remodeling studies is critical to providing new insights for preventing arrhythmogenic atrial remodeling and introducing novel therapies for AF. Likewise, rhythm and rate control strategies in the geriatric population have low efficacy and high mortality. [11]

With this background, we are still in the early stages of understanding the specific role and function of atrial fibroblasts and their impact on AF. In line with this, finding novel therapeutic possibilities in this regard is of interest. Finding a way not only to stop atrial fibrosis but also to reverse this trend could lead to innovative novel approaches for preventive care in the geriatric population or effective therapy for patients suffering from AF. Recently, atrial fibrosis reversal has been proposed as a novel therapeutic strategy in experimental studies. [12] To the best of our knowledge, no therapies have been developed to reverse atrial fibrosis effectively, and much more mechanistic knowledge is essential for this therapeutic innovation.

The aim of this study was to reveal the effects of eplerenone to suppress age-dependent fibrosis, due to the normal aging process and not to specific pathologies like diabetes and hypertension, with a focus on the role of osteopontin as a novel pro-fibrotic marker. In the heart, OPN is associated with the extracellular matrix organization, including collagen and fibronectin formation, and is also involved in angiotensin II–induced fibrosis and could be related to the development of heart failure. [13] Recent studies have reported that OPN could have a pivotal role in cardiac remodeling and fibrosis. [14] Eplerenone is a novel antagonist of the aldosterone receptor, which could decrease coronary artery damage through OPN-related mechanisms and angiotensin II–induced inflammation. [15]

METHODS

Animal Model and Grouping

To evaluate age-induced fibrosis, Fischer-344 (F-344) rats aged 22 to 24 months were

used (Pasteur Institute of Iran). These hybrid rats are offered as a model of age-related changes by the National Institute on Aging and, compared with inbred rats, have a more gradual aging rate and live longer. [16]

All experimental procedures with the animals were confirmed by the Ethics Committee of Rajaie Cardiovascular Medical and Research Center (ID: RHC.AC.IR.REC.1396.4) and were in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

Overall, 45 animals were divided into 3 groups (n=15 rats per each group):

1. Young rats (2–3 months old)
2. Aged rats (22-24 months old) without any treatment
3. Aged rats treated with eplerenone

Eplerenone was administered at 100 mg/kg/day subcutaneously for 2 weeks. [17] Following treatment, the rats were deeply anesthetized with xylazine (10 mg/kg) and ketamine (50 mg/kg). After a mid-sternal incision, the heart was quickly removed, washed in cold normal saline, and atrial tissue was prepared for further studies.

Histological Assessment

Following 2 weeks of treatment, the animals were anesthetized, and the atrial tissues were removed. The tissues were fixed overnight in a 4% paraformaldehyde solution, followed by dehydration and embedding in paraffin. The tissues were then cut into 5-µm thick sections. Masson's Trichrome staining was applied for the detection of collagen fibers in atrial tissues. In the stained sections, the collagen fibers, nuclei, and background (muscle and cytoplasm) were stained blue, black, and red, respectively. Digital images of the stained sections were acquired with a light microscope at 400× magnification (Olympus, Hamburg, Germany). Interstitial and perivascular fibrosis were measured using ImageJ software (http://rsbweb.nih.gov/ij/), and the percentage of fibrosis was calculated.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated from atrial tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and as previously described. [18] The concentration of RNA was measured by a Nanodrop spectrophotometer (Thermo Scientific, USA), and the quality of RNAs was evaluated by gel electrophoresis.

One microgram of total RNA was treated with RNase-free DNaseI (Thermo Scientific, USA) to eradicate any possible traces of DNA contamination. Half a microgram of DNase-treated RNA was then used for reverse transcription, performed with the PrimeScriptII TM Reagent kit (Takara, Japan).

Quantitative PCR (q-PCR) was performed using SYBR Green chemistry with the BIOFACTTM 2X real-time PCR master mix (for SYBR Green I; BIOFACT, South Korea), in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). Briefly, cDNA corresponding to 100 ng of RNA was applied to the SYBR Green ready mix (0.5 µM of each specific primer, 10 µL of SYBR-Green ready mix, and 0.1 µL of Rox) in a total reaction volume of 20 µL.

All real-time PCR reactions were performed in duplicates, and the resultant mean threshold cycles (Ct) were used for further analysis. The thermal profile comprised 95 °C for 30 seconds, 40 cycles of denaturation at 95 °C for 5 seconds, and annealing at 60 °C for 30 seconds. The Ct for individual

reactions was calculated using StepOne software, v2.3.

The expression level of osteopontin in atrial tissues was normalized to that of the internal control, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and relative gene expressions were calculated with the 2ˆ−ΔCt method. The sequences of OPN and GAPDH primers were as follows: OPN; 5′-CCGATGAGGCTATCAAGGTC-3’ (forward) and 5′-ACTGCTCCAGGCTGTGTGTT-3’ (reverse), GAPDH; 5′-CAACTCCCTCAAGATTGTCACCAA-3′ (forward) and 5’-GGCATGGACTGTGGTCATGA-3′ (reverse). The primers for the target genes and GAPDH were purchased from Macrogen Company (South Korea).

Statistical Analysis

The GraphPad Prism, version 6.07 (USA), was used to analyze the data. The results were presented as mean ± standard error of the mean (SEM). Between-group analyses were conducted using one-way analysis of variance (ANOVA), followed by the post hoc Tukey test, and a *P* value ≤0.05 was considered a significant difference.

RESULTS

Eplerenone reversed age-dependent atrial fibrosis.

Perivascular and interstitial fibrosis were evaluated by detecting collagen fibers in the atrial tissues in the study groups. In the young control group, interstitial fibrosis was detected very rarely in the atrial tissues (9.6±1.5%) (Fig. 1A & 1D) compared with the aged control group (47.3±2.5%) (Fig. 1B & 1D). The differences between the young and aged rats were statistically significant (*P*≤0.001).

Two weeks of treatment with eplerenone significantly decreased the percentage of atrial interstitial fibrosis in aged-treated rats (17.1±1.9%) (Fig. 1C & 1D) compared with untreated aged rats (*P*≤0.001).

Perivascular fibrosis in aged control rats (42.4±1.76%) (Fig. 2B & 2D) was significantly higher than in young control rats (7.2±0.67%; *P*≤0.001) (Fig. 2A & 2D). Importantly, eplerenone treatment almost completely abolished the aging-induced perivascular fibrosis in aged-treated rats compared with aged control rats (17.3±0.85%; *P*≤0.001) (Fig. 2C & 2D).

Interstitial and perivascular fibrosis was reversed in the atria of aged rats after treatment with eplerenone.

Eplerenone decreased OPN Expression in aged rats.

To investigate the role of pro-fibrotic mRNA transcripts for OPN and the therapeutic effects of eplerenone in the normal aging process leading to atrial fibrosis, real-time PCR analysis was performed for OPN mRNA. Expression levels of OPN mRNA were significantly upregulated in aged control rats (0.16±0.02) (Fig. 3) compared with young control rats (0.049±0.01; *P*≤0.01) (Fig. 3). Eplerenone treatment significantly downregulated the aging-induced upregulation of OPN mRNA levels compared with untreated aged rats (0.069±0.018; *P*≤0.01) (Fig. 3).

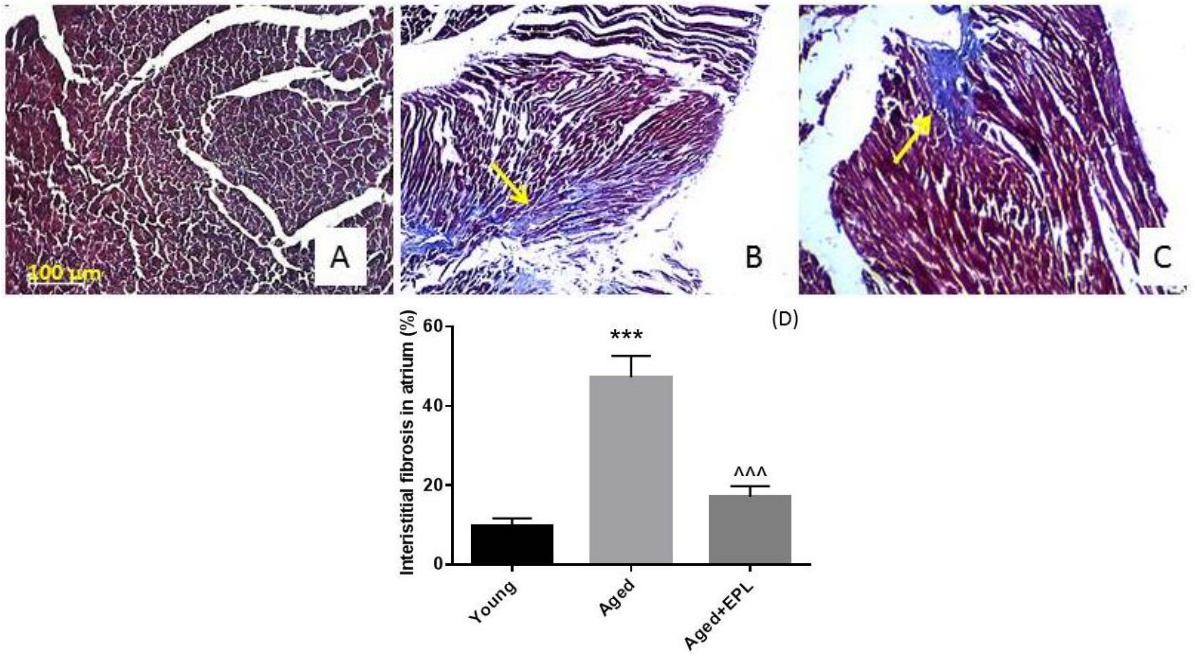

Figure 1: The image depicts interstitial fibrosis in the atria of the young (A), aged (B), and aged+EPL (C) groups (400×). The yellow arrows show interstitial fibrosis (scale bar=100 µm). The results are shown as mean±SEM.
*** *P*<0.001 vs the young group
^^^ *P*<0.001 vs the aged group (D).
EPL: eplerenone

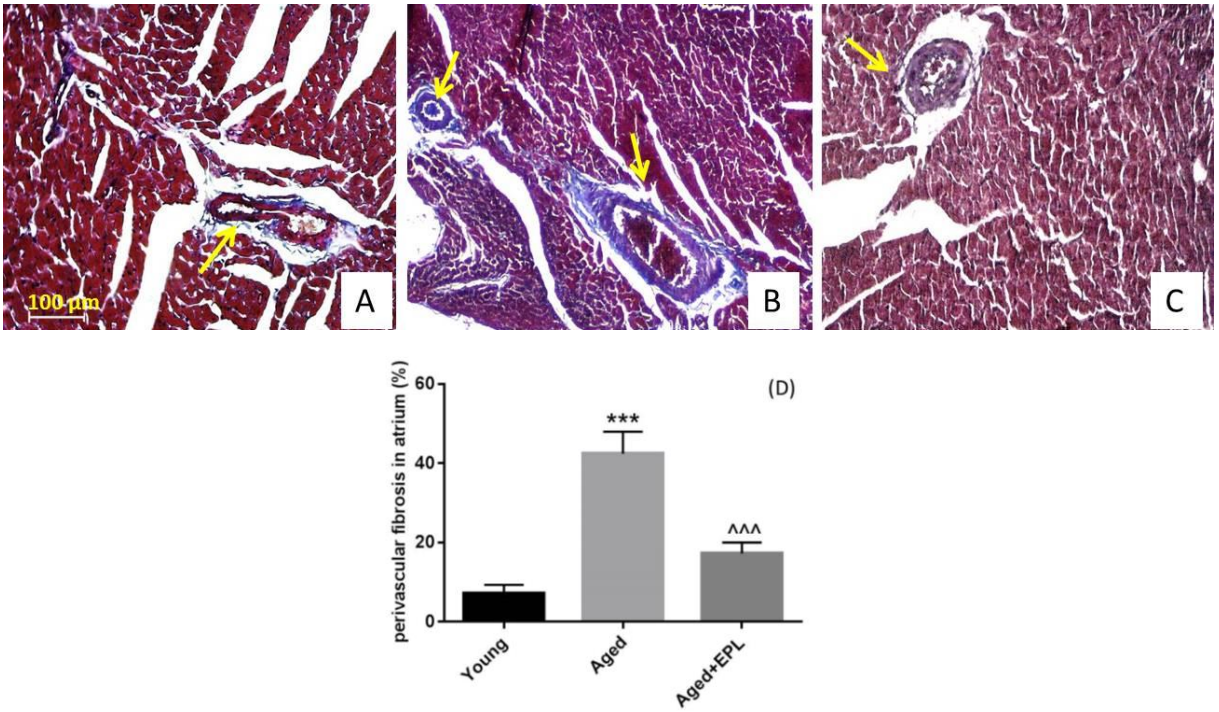

Figure 2: The image demonstrates perivascular fibrosis in the atria of the young (A), aged (B), and aged+EPL (C) groups (400×). The yellow arrows show perivascular fibrosis (scale bar=100 µm). The results are shown as mean±SEM.
*** *P*<0.001 vs the young group
^^^ *P*<0.001 vs the aged group (D).
EPL: eplerenone

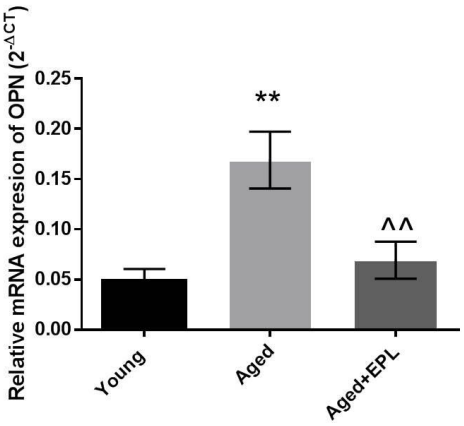

Figure 3: The image shows the expression of osteopontin mRNA and the effects of eplerenone treatment on atrium fibrosis among the aged rats. The results are shown as mean±SEM.
** *P*≤0.01 vs the young group
^^ *P*≤0.01 vs the aged group
EPL: eplerenone; OPN: osteopontin

DISCUSSION

The prevalence of AF is increasing worldwide. Age is a nonmodifiable risk factor for AF. Considering aging in cardiac remodeling studies could be helpful in providing a novel therapy for AF and insights into preventing arrhythmogenic changes. Cardiac fibrosis, causing AF through interrupting fiber continuity, leads disturbed conduction and signal propagation, [19] which is a crucial reason for therapeutic resistance in long-standing AF. [20]

In this study, we demonstrated that the normal aging process induced the development of atrial fibrosis in aged rats compared with young rats. Treatment with eplerenone, a novel antagonist of the aldosterone receptor, inhibited the development of interstitial and perivascular fibrosis in atrial tissue. This result could reveal the etiological role of the aldosterone receptor in the atrial structural remodeling of aged rats.

Based on Masson's Trichrome staining results, aged rat atrial tissue showed substantial interstitial fibrosis compared with young rats. Accordingly, a recent study has shown that interstitial fibrosis could generate early after depolarizations and ectopic pacemakers, which leads to arrhythmias and fibrillation. [21] Our study results also demonstrate that these age-related fibrotic changes could be modifiable and reversible by eplerenone. We showed that the mRNA expression level of OPN in aged rats was significantly higher than in young rats, and eplerenone could effectively downregulate OPN expression. Its expression has been decreased in treated-aged rats compared with untreated-aged rats, and there is no significant difference between eplerenone-treated aged rats and young rats.

Based on these results, OPN has a crucial role in the progression of age-related cardiac fibrosis and atrial structural remodeling. As both tissue fibrosis and OPN expression decreased following treatment with eplerenone, it can be concluded that age-related changes in atrial structure are reversible by inhibiting pro-fibrotic components, despite the fact that we know that age is a nonmodifiable risk factor for cardiovascular disease. For the first time, Henry et al [22] showed that age-induced atrial fibrotic changes could be reversible. They demonstrated that relaxing had extensive

antifibrotic effects in the atria of aged rats by downregulating pro-fibrotic components, such as collagen I, collagen III, and TGF-β.

OPN was initially discovered as a member of bone matrix protein, [23] while more recently, it has been introduced as an important protein involved in cardiovascular diseases, such as acute heart failure, coronary artery disease, and post-MI wound repair. [3, 24] OPN has low basal level expression and may be less significant in normal cardiac tissue. [25] However, in different cardiovascular diseases, the activation of pro-inflammatory and pro-fibrotic factors triggers the upregulation of OPN [26, 27] and could lead to collagen remodeling.

Previously, it has been investigated that cardiac fibrosis could be inhibited by nitric oxide (NO) production, [28] whereas upregulation of OPN suppresses the expression of inducible NO synthase (iNOS) and could develop cardiac fibrosis by decreasing NO production. [29] OPN also could regulate the renin-angiotensin-aldosterone system, which is another contributor to fibrotic remodeling and leads to cardiac hypertrophy, left ventricle dilatation, and myocardial compliance reduction. [30] These data demonstrate the crucial role of OPN in collagen remodeling in the natural aging process. [31]

Based on our obtained results, eplerenone significantly decreased atrial fibrosis in aged rats, and the effect of eplerenone on the inhibition of fibrosis might partially be mediated through the inhibition of OPN expression because treatment of aged rats with eplerenone decreased the mRNA expression of OPN significantly. Recently, an important relationship between OPN expression and angiotensin II-induced coronary artery damage has been suggested, which eplerenone could potentially mitigate.

Limitations of the Study

There are limitations to the methods that we utilized in the study. We suggest examining the expression of osteopontin at the protein level with western blot analysis before and after treatment with eplerenone. In addition, there are other possible targets of eplerenone, such as the mineralocorticoid receptor, through which eplerenone could exert its effect. These factors were not investigated in our study and should be considered in future research.

CONCLUSIONS

Aged rat hearts exhibit more interstitial and perivascular fibrosis compared with young adult rats. Targeting cardiac fibroblast function with the anti-fibrotic drug, eplerenone, could effectively reduce atrial fibrosis by downregulating the pro-fibrotic cytokine, OPN. Eplerenone may also reverse age-related cardiac remodeling through the inhibition of pro-fibrotic elements replacement in the cardiac tissue.

Our findings suggest a novel potential therapy targeting OPN to reduce aging-related cardiac fibrosis in the geriatric population. However, we are not at the threshold of achieving this aim now. Future mechanistic studies and more analyses are necessary to confirm these effects in the near future and introduce an effective and safe therapy to prevent or reduce AF in the healthy geriatric population.

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