EXPERIMENTAL STUDY

Fisetin Alleviates Atrial Inflammation, Remodeling, and Vulnerability to Atrial Fibrillation after Myocardial Infarction

Liang Liu, MS, Shouyi Gan, MB, Bin Li, MS, Xiong Ge, MB, Hui Yu, MS and Huiliang Zhou, MS

Summary

Atrial inflammation and fibrosis are the critical processes involved in atrial fibrillation (AF) after myocardial infarction (MI). Fisetin is a dietary flavonoid that has shown forceful anti-inflammatory and anti-proliferative properties in diverse models of disease. However, fisetin’s role in atrial inflammation, fibrosis, and AF vulnerability post-MI remains completely unknown.

Rats were subjected to MI surgery, by left anterior descending coronary artery ligation or sham operation, and treated with DMSO or fisetin via intraperitoneal injection. After 28 days, echocardiographic parameters were performed, and AF inducibility was tested. We further evaluated the inflammation, fibrosis of left atria (LA), and related signal pathways by RT-PCR, Western blot, and staining analysis.

Compared to the MI group, fisetin treatment improved cardiac function, inhibited macrophage recruitment into the LA and production of IL-1β and TNF-α, and attenuated adverse atrial fibrosis following acute myocardial infarction (AMI). Electrophysiological recordings, using an isolated perfused heart, showed that MI-induced higher inducibility of AF and prolonged AF duration, interatrial conduction time (IACT), atrial effective refractory period (AERP) were significantly alleviated by fisetin. Mechanistically, fisetin markedly increased phosphorylated AMPK (p-AMPK) levels and suppressed NF-κB p65, p38MAPK, and smad3 phosphorylation in the LA post-MI.

We demonstrate that fisetin improves LA expansion, cardiac function, atrial inflammation, fibrosis, and vulnerability to AF following MI by possibly regulating AMPK/NF-κB p65 and p38MAPK/smad3 signaling pathways.

Key words: Fibrosis

Atrial fibrillation (AF) is increasingly recognized as a major global health burden and is obviously associated with the risk of embolic stroke, heart failure, and overall mortality. AF can present as a complication in acute myocardial infarction (AMI); AF in AMI patients can further deteriorate cardiac function and coronary perfusion. The relationship between MI and AF has been described as “AF, a risk marker or a causal mediator of death after MI.”

AF is associated with atrial fibrosis, inflammation, atrial overload, atrial ischemia, ventricular dysfunction, abnormal ion current, and channel expression. Among these, the pathophysiology of AF post-MI have focused on the inflammation and fibrosis of the atria. Inflammatory cell infiltration has been observed in atrial tissue of AF; moreover, some inflammatory markers, such as CRP, IL-1β, and TNF-α, were closely associated with the origin of AF by promoting arrhythmic substrate, including automaticity, altered Ca²⁺ handling, conduction slowing, hypertrophy, and fibrosis. Further studies considered anti-inflammatory therapy as one of the candidates to inhibit AF and improve the mortality of AMI. Atrial fibrosis that is linked, not only to stimulation of myocytes and fibroblasts, but also to the activation status of inflammatory cells, has also emerged as an important pathogenic contributor of AF initiation, progression, and maintenance by reentry activity and slow conduction. Inhibition of myocardial fibrosis and oxidative stress can reduce AF occurrence. These causes suggests that suppressing the process of atrial inflammation and fibrosis is a potential therapeutic target of AF.

Fisetin (3, 3, 4, 7-tetrahydroxyflavone) is a dietary flavonoid found in various fruits, vegetables, nuts, and wine, and is easily absorbed and distributed to the blood vessels. Excessive evidence has demonstrated that fisetin could inhibit various chronic inflammation-related conditions, cell proliferation, oxidative stress, and cardiac hypertrophy. Meanwhile, fisetin significantly attenuates the ischemia/reperfusion (I/R)-induced myocardial tissue injury by suppressing mitochondrial oxidative stress, and...
decreases infarct size after transient cerebral middle artery occlusion by inhibiting inflammation.\textsuperscript{18,19} However, whether fesitin exerts helps suppress the progression of atrial inflammation and fibrosis related to vulnerability to AF in the MI model remains unknown.

Based on these reasons, we explored fisetin’s role in atrial remodeling progression by measuring atrial inflammation and interstitial fibrosis, which somehow simulates the AF pathology associated with MI, and AF inducibility in the rat model of MI.

Methods

Rat model of MI: All animal experiments were conducted in accordance with the guidelines of the National Institutes of Health (the 8th Edition, NRC 2011), Male Sprague-Dawley rats (SD) (180-220 g) were randomly divided into the following three groups: Sham, MI, Fisetin + MI group. The MI model was made by ligating of the left anterior descending coronary artery as previously described.\textsuperscript{20} In the Fisetin + MI group, rats received a daily intraperitoneal injection of fisetin (50 mg/kg, TOCRIS Bioscience, Bristol, UK) from seven days before surgery to 28 days after MI surgery.\textsuperscript{17,19} The Sham and MI groups were administered the same volume of placebo (DMSO).

Echocardiographic assessment: In order to evaluate left ventricular (LV) function and left atrial diameter (LAD) in the rat model of MI.

Quantitative RT-PCR: Total RNA of left atrial (LA) tissues was extracted by use of TRIzol Reagent (Invitrogen, Carlsbad, CA), single-stranded cDNA was transcribed with the help of the PrimeScript\textsuperscript{TM} RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The transcript level was measured relative to that of GAPDH using a calibration curve. The PCR primers from the rat are listed in the Table I.

Western blot analysis: Total proteins were isolated from LA tissues with lysis buffer. Proteins of interest LA tissues were separated on SDS-PAGE gels, transferred to PVDF (Millipore, Hong Kong, China) membranes, and incubated with appropriate following primary antibodies: IL-1β, TGF-β1, α-SMA, Smad2/3, p-Smad3, p38 MAPK, p-AMPK, AMPK, and NF-κB p65 before programmed electrical stimulation, and were then analyzed using the Quantity One analysis system software (Bio-Rad Laboratories, UK).

Masson’s trichrome staining: The sections of LA tissues (5 mm thickness) were fixed with 4% paraformaldehyde, then dehydrated and embedded in paraffin. To evaluate the degree of cardiac fibrosis, the fraction of fibrosis area as the percentage of fibrosis area (blue staining) to the total LA area in an average of five sections of each heart was measured using the Masson’s trichrome staining. The image was analyzed at 400 × magnification using the image analysis system software (Image-Pro-Plus version 6.0).

Immunohistochemistry and immunofluorescence staining: Immunohistochemical IL-1β staining of LA sections were performed using anti-rat IL-1β antibodies (Abcam) with a dilution factor of 1:200. The sectioned tissues were subsequently incubated with biotinylated goat anti-rabbit secondary antibody. Images (× 400 each field) were visualized by light microscopy.

Sections were incubated with diluted anti-rat CD68 antibodies (1:1000; Servicebio technology, Wuhan, China), subsequently, secondary antibodies conjugated with fluorescence were incubated. DAPI (1:1; Servicebio technology, Wuhan, China) was for nuclear visualization, images (× 400 each field) were captured using an Olympus fluorescence microscope.

Surface electrocardiogram (ECG): ECG recordings were performed, under anesthesia, with isoflurane (1.5% volume in oxygen). Three lead surfaces of ECG were recorded from subcutaneous needle electrodes attached to each limb and the simulated limb lead II, ECG parameters (Heart rate, Q wave duration, and PR interval) were analyzed using LabChart 7 Pro-analysis software (AD Instruments).

Electrophysiologic studies: As described previously,\textsuperscript{22,24} electrophysiological studies were performed on day 28 using isolated perfused hearts using a Langendorff apparatus with HEPES-buffered Tyrode’s solution (mM: NaCl 130; KCl 5.4; CaCl2 1.8; MgCl2 1; Na2HPO4 0.3; HEPES 10; glucose 10; pH adjusted to 7.4 with NaOH) equilibrated with a 95% O2/5% CO2 gas mixture at 37 °C and at a constant pressure of 60 mmHg. All isolated hearts were stabilized for 10 min by perfusion at a constant velocity, before programmed electrical stimulation, and were then

---

**Table I. Information of Primers for RT-PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward Sequence (5’-3’)</th>
<th>Reverse Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>ACCCGGAGACGACAATCACAC</td>
<td>CTTGGTGCCGTACAGATGGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GTGGCAGCTATCTATGTGTCG</td>
<td>CCACTTGTTGCTATGTGTCG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GTGATCGTCTCCCAABAAAGGA</td>
<td>AAGGTACCGGTCGGCTGGA</td>
</tr>
<tr>
<td>collagen I</td>
<td>CCGTGACCCCAAAGATGTCGG</td>
<td>GAACCTCTGCTTCATACTCG</td>
</tr>
<tr>
<td>collagen III</td>
<td>GACCTTCTGGAAGATTGATCC</td>
<td>AAATCCATGGATCTACCCCG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGCTAACATCAAATGGGGTG</td>
<td>TTGCTGAACATCAAATGGGGAG</td>
</tr>
</tbody>
</table>

---

Int Heart J
November 2019
FISETIN REDUCES ATRIAL FIBRILLATION VULNERABILITY

I n tH e a r tJ

Beverly, MA, USA, AMPK (CST), p-NF-κB, p38, MAPK (Santa Cruz), P-Smad3 (Bioworld, Minneapolis, MN, USA), Smad2/3 (Abcam), GAPDH (Abcam), followed by horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands were detected by chemiluminescence (ECL) and were visualized using a Kodak Image Station 4000 (Rochester, NY). The bands’ densities were quantified using the Quantity One analysis system (Bio-Rad Laboratories, UK).
stimulated with a pair of electrodes placed on the right atrium. Two sets of silver bipolar electrodes were placed on the appendages of the right atria (RA), LA. The distance between two electrodes was set at 10 mm to measure the interatrial conduction time (IACT) during right atrial regularly pacing (S1 × 10). The effective refractory period (ERP) of the LA and RA was measured through the S2 extra-stimulus method based on regularly paced beats (S1 × 8) with basic cycle lengths (BCLs) of 150, 120, and 90 ms. AF inducibility was tested by applying 3-second burst pacing methods at CLs of 50-20 ms (10 ms decrement, pulse duration = 5 ms). AF was defined as rapid and irregular atrial excitations lasting at least 1 second. The duration of AF was measured from the end of burst pacing to the first P wave detected after the rapid irregular atrial rhythm.

**Statistical analysis:** Statistical analysis was performed using SPSS 22.0 software. Continuous variables were tested for normal distribution, using one-way ANOVA, followed by the Tukey post-test expressed as mean ± SEM. and the Kruskal-Wallis test was used for non-normally distributed variables expressed as medians and interquartile ranges. Differences within groups were analyzed by student’s independent t test or Mann-Whitney U test. AF incidence across groups was analyzed using Fisher exact test expressed as percentages. *P-value < 0.05 was considered statistically significant.

**Results**

**Fisetin attenuates cardiac dysfunction and reduces cardiac enlargement after MI:** As shown in Table II, the heart rate (HR) of MI-induced rats remained significantly unchanged, although having the trend of descent, for four weeks. Seemingly, fisetin could increase HR after MI, but it was not statistically significant. The cardiac dimensions and LV function were measured by echocardiography before terminal experiments; there were clear differences among the three groups. Enlarged LAD and LVEDD, and reduced LVEF and LVFS, were presented in MI group. Statistical analysis was performed using SPSS 22.0 software. Continuous variables were tested for normal distribution, using one-way ANOVA, followed by the Tukey post-test expressed as mean ± SEM. and the Kruskal-Wallis test was used for non-normally distributed variables expressed as medians and interquartile ranges. Differences within groups were analyzed by student’s independent t test or Mann-Whitney U test. AF incidence across groups was analyzed using Fisher exact test expressed as percentages. *P-value < 0.05 was considered statistically significant.

**Table II.** Effect of Fisetin on HR and Echocardiographic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n = 8)</th>
<th>MI (n = 8)</th>
<th>Fisetin (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>423 ± 9</td>
<td>405 ± 10</td>
<td>420 ± 14</td>
</tr>
<tr>
<td>LAD (mm)</td>
<td>3.85 ± 0.31</td>
<td>7.21 ± 0.29*</td>
<td>4.58 ± 0.21*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>7.26 ± 0.19</td>
<td>11.10 ± 0.22*</td>
<td>9.14 ± 0.17*</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>74.2 ± 1.8</td>
<td>48.9 ± 2.3*</td>
<td>61.2 ± 1.7*</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>43.1 ± 2.4</td>
<td>21.3 ± 1.8*</td>
<td>34.9 ± 1.5*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (n = 7 per group). *P < 0.05, versus Sham. +P < 0.05, versus MI.

**AF inducibility and electrophysiological properties:** Representative ECG recordings, after an ex vivo burst pacing using an isolated perfused heart, was presented in Figure 3A. Figure 3B indicated AF inducibility; there was less incidence of AF in sham group (1/11, 9.09%). In contrast, AF inducement was significantly higher (9/11, 81.81%) and subjected to MI for 28 days, which was suppressed by fisetin treatment (2/11, 18.18%). The analysis of AF duration is shown in Figure 3C. AF duration was significantly longer in the MI group compared with the Fisetin+MI group (median [25%-75% interquartile range], 6.96 [0.12-35] versus 0 [0-0.13] seconds).

Besides, the duration of the P wave and PR interval were remarkably prolonged in the MI group compared with the Sham group (Figure 3D and E); however, fisetin decreased the extension of P wave duration and PR interval. Compared with the sham group, IACT, ERP of LA, and RA in the MI group were prolonged at all the BCLs (90, 120, 150 ms) tested; these prolongations were attenuated by treatment with fisetin (Figure 3F-H).

**Fisetin inhibits MI-induced atrial inflammation and fibrosis by regulating AMPK/NF-κB and MAPK/Smad3 signaling pathways:** To clarify the potential molecular mechanism related to fisetin’s effects on MI-induced atrial inflammation and fibrosis, we detected the well-known macrophage specific marker (CD68) at 28 days post-MI. Compared with the sham group, the number of CD68-positive macrophages infiltrating the LA was increased in the MI group. Similar results were obtained from quantitative analysis of mRNA expression of CD68. Fisetin could alleviate the infiltration of CD68+ macrophages (Figure 1A-C), and the mRNA levels of molecules related to pro-inflammatory signals, as shown in Figure 1D and E. The results presented indicated that the increased expression of IL-1β and TNF-α after MI was decreased by fisetin treatment. The immunohistochemical result of IL-1β also demonstrated the effect of fisetin’s anti-inflammatory response. The expression of IL-1β in the LA was increased in the MI group, fisetin resulted in significant reduction of IL-1β (Figure 1F and G). We further illustrated, by Western blot analysis, that fisetin decreased IL-1β expression (Figure 1H).

**Fisetin attenuates MI-induced atrial fibrosis:** As shown in Figure 2A and B, the quantitative ratio of the area of fibrosis to the area of the total LA tissue by masson trichrome staining showed increased fibrosis at 28 days post-MI. However, fisetin remarkably decreased deterioration of atrial interstitial fibrosis. RT-PCR analysis showed the deposition of cardiac collagen I and III was elevated in the MI group, which was significantly downregulated by fisetin (Figure 2C and D). Meanwhile, Western blot confirmed the results that significant upregulation in α-SMA, TGFβ1 and CTGF protein expression was found in the MI group, whereas fisetin significantly reduced the expression of these indexes (Figure 2E-G).

**Discussion**

Fisetin could alleviate the infiltration of CD68+ macrophages and TNF-α, and further enhanced AMPK phosphorylation, as indicated by Western blot analysis. The ratio of phosphorylated to total NF-κB p65, p38MAPK, and smad3 protein expression were elevated in LA tissue, while fisetin markedly suppressed these effects (Figure 4B-D).
Figure 1. Infiltration of macrophage and left atrial inflammation. Representative immunofluorescence images (A) and quantitative analysis (B) on CD68 positive (CD68+) expression in left atria (LA) sections (n = 4, per group). The corresponding expression level of CD68 mRNA was analyzed by RT-PCR method (C) (n = 3, each group). D, E: The mRNA levels of IL-1β, TNF-α (n = 3, each group). Representative immunohistochemistry images (F) and quantitative analysis (G) of IL-1β staining (n = 4, per group). H: Western blot analysis for the protein expression of mature IL-1β (n = 3, per group). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 for indicated comparisons.
Discussion

In the present study, the potential protective functions of fisetin on atrial remodeling were detected at four weeks in the post-MI rat model. The results demonstrate that fisetin ameliorated the arrhythmogenic substrate by reversing cardiac dysfunction, atrial expansion, inflammation and fibrosis, and finally improved AF vulnerability, atrial conduction function and shorten atrial ERP (AERP), possibly through regulation of the AMPK/NF-κB and MAPK/...
Smad3 signaling pathways. Therefore, fisetin may be a promising effective drug to inhibit AF incidence after MI.

AF origin and maintenance are closely associated with atrial inflammation, fibrosis, LA expansion, and cardiac function following MI, which has been demonstrated in AF patients and animal models.6,26,27 Due to fisetin’s activities with various biological effects of anti-proliferation and anti-inflammation, especially mediated by macrophages,16,28 we inferred that fisetin plays an inhibitory role in atrial inflammation and fibrosis after MI. To verify the hypothesis, we examined the effects of fisetin on inflammation, fibrosis, and partial electrophysiological properties of atria after MI. Consistent with previous results, fisetin alleviated macrophage infiltration and pro-inflammatory cytokine production while exacerbating atrial overload.28,29 Meanwhile, fisetin also downregulated α-SMA as a marker of CFs differentiation into myofibroblasts and profibrotic stimulators, TGFβ1 and CTGF, which con-

Figure 3. Recordings of electrocardiogram (ECG) in vivo and electrical activity ex vivo. A: Representative ECG of atrial fibrillation (AF), induced by atrial burst pacing in isolated perfused hearts, from the MI group (n = 11, per group). B, C: AF inducibility and AF duration. D, E: The duration of P wave, PR interval were analyzed (n = 8, per group). F-H: IACT, ERP of the LA and RA were measured at basic cycle lengths (BCLs) of 150, 120, and 90 ms (n = 8, per group). SR indicates sinus rhythm.
Figure 4. Effects of fisetin on protein relating to pro-inflammatory and profibrotic signals. Representative Western blot and quantitative analysis of phosphorylated protein ratios of AMPK (A), NF-κB p65 (B), p38MAPK (C) and smad3 (D) from LA post-MI (n = 3, per group). P-indicates the phosphorylated form of the protein. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 for indicated comparisons.

firmed that fisetin can similarly inhibit inflammation and fibrosis in the atria after MI. Although fisetin can improve hemodynamic disorder in the ischemia/reperfusion (I/R) injury rat model, the study did not directly detect cardiac function parameters by echocardiography. In the present study, we demonstrated that fisetin could improve LA expansion and cardiac function of long-term MI.

A previous study revealed that inflammatory stimulation increases AF incidence and aggravates atrial electrical remodeling by downregulating the level of L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) and Ca\(^{2+}\) gene, but macrophage depletion can partly restore them.\(^{11,10}\) Inflammation, including pro-inflammatory cytokines TNF-α and IL-1β, also can change repolarizing K\(^{+}\) current.\(^{31,32}\) These changes in expression and current of ion channels in atria could be considered one of mechanisms underlying atrial tachyarrhythmia in inflammatory diseases. The fiber bundle’s electrophysiological effect mainly causes a physical barrier to conduction, which ultimately results in local conduction heterogeneity during the indexes of electrophysiological research. The prolongation of IACT and P wave duration usually represents the interatrial block and enlarged atria, while fibrosis inhibition may explain subdued atrial ERP and IACT prolongation.\(^{29,33}\) Recent research, focused on exploring the role of anti-inflammatory and anti-fibrotic treatment, have approved AF prevention following MI,\(^{33,34}\) but we also can’t ignore the effect of reducing AF by improving atrial expansion and cardiac dysfunction. Regard-
ing the relationship between AF and AERP, some studies considered that AERP was shortened in some models, including rapid atrial pacing and early phase of MI (< 45 minutes) model, but AERP was prolonged in long-term MI model (four weeks). Therefore, the result of IACT and AERP prolongation we obtained in the long-term MI model was reasonable. Interestingly, shortening the PR interval suggested that fisetin can improve atrioventricular conduction. Based on previous research, the referred effects of fisetin on prolongation of IACT, AERP, P wave duration, PR interval, and AF vulnerability may be because of inhibited LA expansion, macrophage infiltration, secretion of TNF-α and IL-1β and fibrosis in the atrial tissue and ameliorating cardiac function after MI.

We know that AMPK is considered a cardiac metabolism regulator. AMPK and nuclear factor-xB (NF-xB) can regulate inflammatory gene expression in many diseases, including hypoxia and MI pathology of heart, enhancing AMPK phosphorylation. Attenuating NF-xB phosphorylation can inhibit inflammatory response after tissue ischemia. Moreover, TGF-β/Smad signaling plays an important role in fibrosis, and Smad3’s key role in TGF-β1-induced redundant accumulation of extracellular matrix (ECM) is comprehensively recognized. Meanwhile, activation of mitogen-activated protein kinase (MAPK), including the ERK1/2, JNK1/2 and p38 pathways, can be involved in the pathological process of cardiac fibrosis as an upstream signaling molecule of Smad2/3. In recent years, more attention has been paid to the MAPK and Smad signaling pathway as an effective target of anti-fibrotic therapy. Fisetin treatment can alleviate hepatic lipid metabolism by increasing AMPKα phosphorylation. Meanwhile, fisetin inhibits inflammatory responses by decreasing NF-xB phosphorylation. Fisetin also protects against cardiac hypertrophy, suppressing the MAPK signaling pathway in a cardiomyocyte hypertrophy model. Similarly, in the present study, we observed that fisetin enhanced AMPK phosphorylation in atria after MI, and significantly attenuated phosphorylation of NF-xB p65, p38MAPK, and Smad3 in LA tissues, suggesting that fisetin exerts beneficial effects on atrial inflammation and remodeling post-MI, possibly attributed to partial activation of the AMPK and inactivation of the p-NF-xB p65, p-p38MAPK, and p-smad3 pathway.

Conclusions

Taken together, we demonstrated that fisetin improved AF vulnerability, LA expansion, and cardiac dysfunction, reversed atrial inflammation, especially the infiltration of macrophages, and fibrosis, post-MI, possibly by regulating the AMPK/NF-xB and MAPK/Smad3 signaling pathways. These findings suggest that fisetin is a potential therapeutic drug in treating MI-induced AF.

Disclosure

Conflicts of interest: The authors declare no conflicts of interest. All authors have read and approved the final manuscript.

References

5. Maisel WH, Stevenson LW. Atrial fibrillation in heart failure: epidemiology, pathophysiology, and rationale for therapy. Am J Cardiol 2003; 91: 2D-8D.
failure via the GLP-1 receptor through the eNOS/cGMP/PKG pathway. Peptides 2017; 90: 69-77.