

## Cardiac and renal fibrosis and oxidative stress balance in lipopolysaccharide-induced inflammation in male rats

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### Original Article

#### Abstract

**BACKGROUND:** Subclinical inflammation induced by persistent exposure to lipopolysaccharide (LPS) is found in some clinical conditions such as obesity or diabetes. This study aimed to investigate the effect of recurrent LPS exposure on inflammatory markers, oxidative stress balance and cardiac and renal fibrosis in male rats.

**METHODS:** Male Wistar rats were divided into control and LPS-treated. LPS (10 mg/kg/week) was injected intraperitoneally. After 4 weeks, left ventricles and kidneys were homogenized and stained with hematoxylin and eosin (H&E) and Masson trichrome for histological examination. Serum levels of nitrite, interleukin 6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were measured and total thiol, malondialdehyde (MDA), superoxide dismutase (SOD) and catalase were evaluated in the heart and kidney homogenates.

**RESULTS:** Serum inflammatory markers were higher in LPS group than control (nitrite:  $37.0 \pm 2.2$  vs.  $25.5 \pm 1.9$   $\mu\text{mol/l}$ ; IL-6:  $84 \pm 3$  vs.  $98.0 \pm 4.4$   $\text{pg/ml}$ ; TNF- $\alpha$ :  $75.5 \pm 4.9$  vs.  $85.3 \pm 4.7$   $\text{pg/ml}$ ; respectively,  $P < 0.050$ ). Evaluation of total thiol concentration (heart:  $10.0 \pm 0.9$  vs.  $22.5 \pm 1.2$ ; kidney:  $7.0 \pm 0.5$  vs.  $27.8 \pm 3.1$   $\text{nmol/g tissue}$ , respectively), catalase (heart:  $0.18 \pm 0.03$  vs.  $0.66 \pm 0.04$ ; kidney:  $0.17 \pm 0.03$  vs.  $0.73 \pm 0.03$ ,  $\text{U/g tissue}$ , respectively) and SOD (heart:  $8.01 \pm 0.70$  vs.  $12.3 \pm 0.4$ ; kidney:  $7.02 \pm 0.60$  vs.  $12.0 \pm 0.2$ ,  $\text{U/g tissue}$ , respectively) showed lower levels in LPS-treated group compared to control; while MDA concentration in LPS group was higher than control ( $P < 0.05$ ). Histopathological examination in LPS-treated group indicated infiltration of inflammatory cells and more collagen deposition in left ventricle wall and kidney compared to control group.

**CONCLUSION:** We concluded that in clinical conditions with chronic LPS, cardiac and renal fibrosis occurs even in absence of preceding tissue injury due to imbalances in oxidative stress.

**Keywords:** Inflammation, Lipopolysaccharide, Oxidative Stress, Heart, Kidney

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#### Introduction

Inflammation is a part of complex biological and adaptive immune response of body tissues to harmful stimuli such as irritation that plays a central role in metabolism in a variety of organisms.<sup>1</sup> Inflammatory abnormalities are demonstrated in allergic reactions and some pathological conditions. Moreover, in some diseases including cancer, obesity, atherosclerosis, and ischemic heart disease, inflammatory processes are the origin of etiology.<sup>2-5</sup>

Inflammation is classified as acute or chronic. Acute inflammation is a short-term process, appears within a few minutes or hours to remove injurious

stimulus.<sup>6</sup> Chronic inflammation can be caused by excessive calorie consumption, elevated blood glucose levels, and oxidative stress. The danger of chronic low grade inflammation is its silent nature with destructive power and characterized by increasing in the systemic concentrations of cytokines such as interleukins (ILs), C-reactive protein (CRP) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>7</sup> Several clinical observations indicated that chronic low-grade inflammation contributes in the pathogenesis of many diseases, including obesity, atherosclerosis and cardiovascular disease (CVD).<sup>8-11</sup>

The murine endotoxin or lipopolysaccharide

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(LPS) model is a known model of inflammation which is important in understanding the inflammatory response. LPS, an endotoxin of gram-negative bacteria, via toll-like receptor 4 (TLR4), induces inflammation in the rodent.<sup>12</sup> Heart can be used as a target tissue for TLR4 receptor.<sup>13</sup> The effect of gram negative bacteria on myocardium has been reported in human and different animal models of inflammation or bacteremia.<sup>14-16</sup> It is indicated that low levels of LPS activate cardiac myocytes and depress cardiac contractility as well as apoptosis by activating the renin angiotensin system. On the other hand, recurrent exposure to subclinical LPS may alter left ventricular structure and function.<sup>17</sup> Therefore, we hypothesized that chronic exposure to low level of LPS may induce cardiac and kidney fibrosis.

In the present study, we used a chronic low grade inflammation model, which is very close to clinical conditions with chronic low-grade inflammation, by recurrent exposure to LPS, and evaluated its effects on serum inflammatory markers and cardiac fibrosis in male rats.

### Materials and Methods

Twenty male Wistar rats (8 weeks old; 200-220 g weight) were purchased from animal house of Mashhad University of Medical Sciences, Mashhad, Iran. The animals were kept under standard conditions (temperature  $22 \pm 2$  °C and 12h light/dark cycle) with free access to food and water. Working with the animals was performed in accordance with approved animal protocols and guidelines established by local ethical committee on animal research. The animals were divided into two experimental groups: control and LPS-treated ( $n = 10$  in each group).

LPS was purchased from Sigma (Sigma chemical Co., USA) and dissolved in physiological saline solution. For induction of subclinical inflammation, the rats received LPS (10 mg/kg/week) intraperitoneally for 4 weeks.<sup>18</sup> Control group received 2 ml/kg/week of saline instead of LPS. After 4 weeks, the animals were anaesthetized with diethyl ether and blood samples were taken for further analysis. Then, they were sacrificed, and left ventricle of hearts and right kidneys were dissected, washed with cold saline and kept in 10% formalin solution for histological examination. Right ventricles and left kidneys were stored at -70 °C for tissue marker measurements. Serums were separated by blood centrifugation at 3000 g for 20 min. Then, the samples were stored at -70 °C for further analyses.

Heart and kidney tissues were homogenized in phosphate-buffered saline (PBS) buffer and the supernatant was used for enzyme-linked immunosorbent assay (ELISA). Levels of nitrite in the tissue homogenates and serum were measured using griess reagent method (Promega Co., USA) as previously described.<sup>19-21</sup> Serum IL-6 and TNF- $\alpha$  levels were measured with standard ELISA kits (ebioscience Co., San Diego, CA, USA) according to the manufacturer instructions. The techniques were conducted using automated ELISA Reader (BioTek Instruments, USA). Tissue's total thiol content, malondialdehyde (MDA), and the activity of superoxide dismutase (SOD) and catalase were measured in homogenate solutions.

MDA which is the index of lipid peroxidation was measured as previously described.<sup>22</sup> 2 ml from reagent of thiobarbituric acid (TBA)/trichloroacetic acid (TCA)/ Hydrochloric acid (HCl) and 1 ml of tissue homogenate were mixed. Then, they were centrifuged within 3000 $\times$ g and the absorbance was measured at 535 nm.

For measurement of total thiol concentration, 1 ml of tris-ethylenediamine tetraacetic acid (EDTA) buffer (pH = 8.6) was added to tissue homogenate and absorbance was read at 412 nm against tris-EDTA (TE) buffer alone. Then, 20  $\mu$ l of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added to solution and kept at room temperature for 15 minutes. Then, absorbance was read again. The absorbance of DTNB reagent was also read as a blank.<sup>23</sup>

SOD and catalase activities were measured using a Ransod kit (Randox Laboratory, UK) by a method which was previously described.<sup>24</sup>

The left ventricles of heart and right kidneys were put in 10% buffered formalin for 24-72 hours prior to processing and paraffin following a routine procedure. Then, samples with 5 $\mu$ m thickness were prepared and stained with hematoxylin and eosin (H&E) and Masson's trichrome, and were examined by light microscopy. Fibrosis was quantified in five fields per animal, and expressed as the percentage of fibrous tissue area stained with Masson trichrome. The fibrotic area was quantified using NIH image software (ImageJ).

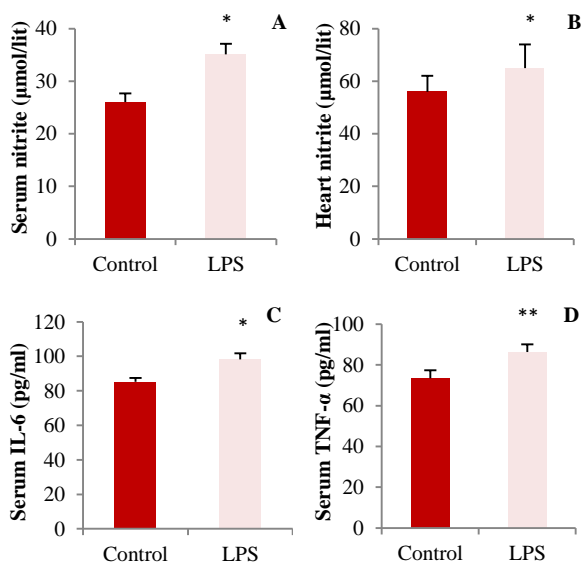
All data were expressed as means  $\pm$  standard error (SE). The data was analyzed using the SPSS software (version 20, IBM Corporation, Armonk, NY, USA). Independent sample t-test was used for comparison of data between groups. Differences were considered statistically significant when  $P < 0.050$ .

## Results

**Body weight:** There was no significant difference in initial body weight between the groups. Over the course of the 4 weeks study, the animals gained body weight, and LPS-treated group gained less body weight compared to the normal group ( $230.0 \pm 5.2$  vs.  $245.4 \pm 3.7$  g) ( $P < 0.050$ ).

**Effect of recurrent LPS administration on serum and heart nitrite levels:** As shown in figure 1, significant increase in serum nitrite concentrations were observed in LPS group as compared to control ( $37.0 \pm 2.2$  vs.  $25.5 \pm 1.9$   $\mu\text{mol/l}$ ; respectively,  $P < 0.050$ ). Nitrite levels in the heart tissue of LPS-treated group showed a higher level than control, although it was not statistically significant ( $P = 0.090$ ).

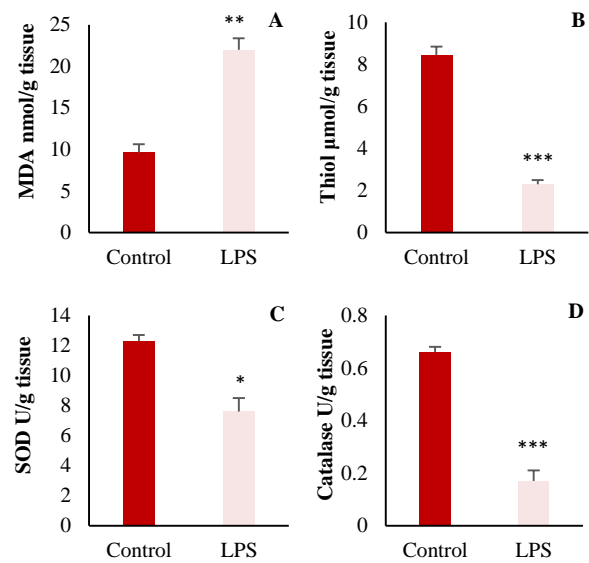
**Effect of LPS administration on serum IL-6 and TNF- $\alpha$  levels:** Figure 1 illustrated the effect of LPS administration on serum IL-6 and TNF- $\alpha$  levels. Serum TNF- $\alpha$  and IL-6 concentrations were significantly higher in LPS-treated group compared to control (IL-6:  $84 \pm 3$  vs.  $98.0 \pm 4.4$ ; TNF- $\alpha$ :  $75.5 \pm 4.9$  vs.  $85.3 \pm 4.7$  pg/ml; respectively, ( $P < 0.050$ ).



**Figure 1.** A: Serum nitrite; B: Heart tissue nitrite; C: Interleukine (IL)-6; and D: Serum tumor necrosing factor (TNF)- $\alpha$  concentrations in control and lipopolysaccharide (LPS)-treated groups, (\*  $P < 0.050$  and \*\*  $P < 0.010$  compared to control, n = 10 in each group)

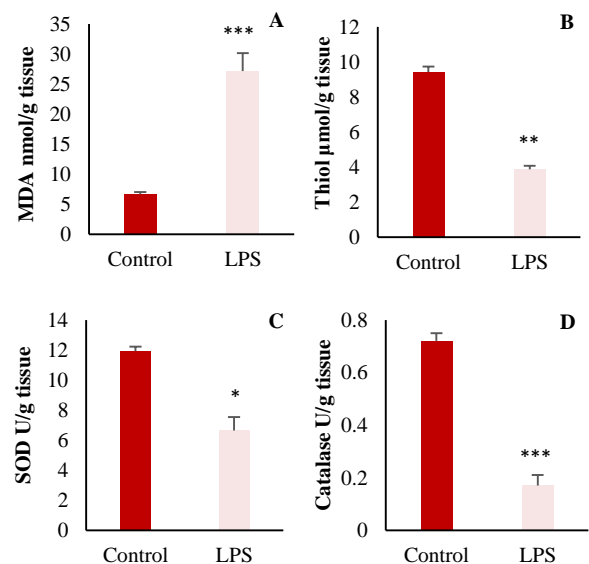
**Tissue oxidant and antioxidant factor:** As shown in figure 2, MDA contents in heart tissues of LPS-treated group were significantly higher than control ( $P = 0.010$ ), while, total thiol concentration,

catalase and SOD were significantly reduced after LPS treatment ( $P = 0.010$ ).



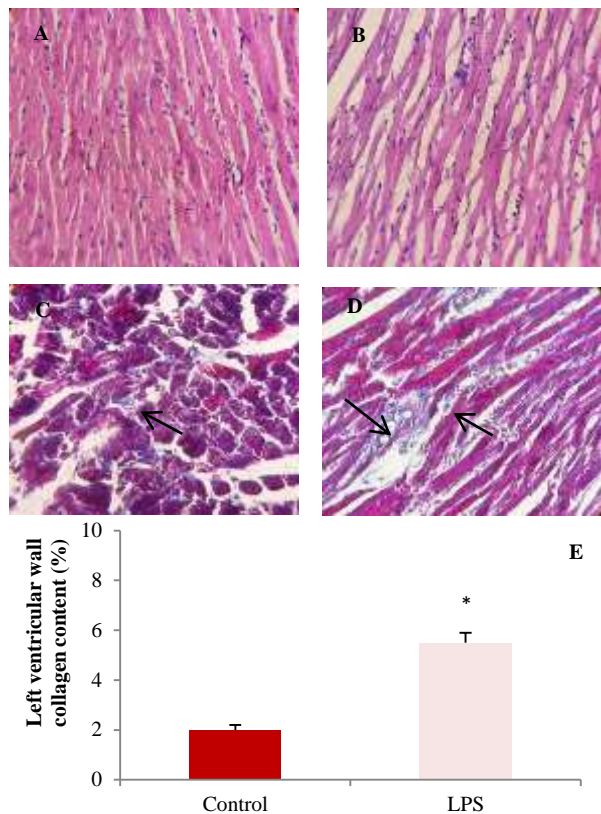
**Figure 2.** Comparison of A: malondialdehyde (MDA); B: Total thiol concentration; C: Superoxide dismutase (SOD); and D: Catalase in the heart of control and lipopolysaccharide (LPS)-treated groups, (n = 10 in each group, \*  $P < 0.050$ ; \*\*  $P < 0.010$ ; \*\*\*  $P < 0.001$  compared to control group)

The same results were also observed in oxidant and antioxidant markers in the kidney (Figure 3).



**Figure 3.** A: Malondialdehyde (MDA); B: Total thiol; C: Superoxide dismutase (SOD); and D: Catalase in the kidney (\*  $P < 0.050$ ; \*\*  $P < 0.010$ ; \*\*\*  $P < 0.001$  compared to control group)

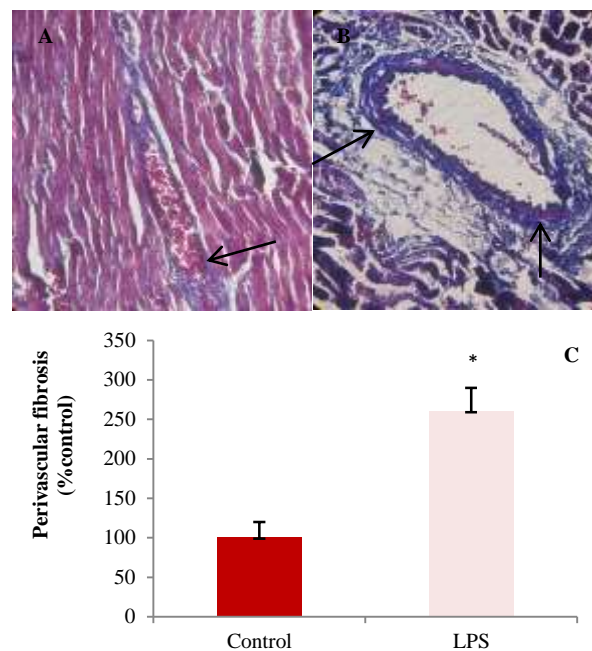
**Histopathological findings:** Figures 4-6 illustrate representative examples of photomicrographs of H&E and Masson tichrome stained of the left ventricular tissues and kidneys in control and LPS-treated groups.



**Figure 4.** The light micrograph of left ventricles in control and lipopolysaccharide (LPS)-treated groups. A: Control group with normal architecture [ $\times 20$ , hematoxylin and eosin (H&E)]. B: Recurrent LPS treatment [(10 mg/kg/week for 4 weeks ( $\times 20$ , H&E))] showing infiltration of inflammatory cells, edema and disarrangement of fibers. Masson trichrome staining of left ventricular muscles of control (C) and LPS (D) groups show more collagen deposition (blue color, black arrows) in LPS-treated group. Blue color indicates collagen fibers. E: Left ventricular wall fibrosis showed higher collagen content (%) in LPS group compared to control (\*  $P < 0.050$  compared to control).

As illustrated in figure 6, the cardiac muscle fibers in control animals have normal histopathological features with single, oval and centrally located nuclei of cardiomyocytes and regularly arranged myofibers. The left ventricle of LPS-treated group showed infiltration of inflammatory cells, disarrangement of fibers, edema, and dilation of blood vessels. Masson's trichrome stained samples showed a significant increase in

collagen deposition in left ventricle wall of LPS group compared to control (Figure 5).



**Figure 5.** Perivascular fibrosis around left anterior descending coronary artery stained by Masson trichrome. Blue color indicates collagen deposition. A: Control; B: Lipopolysaccharide (LPS); C: Percent of perivascular fibrosis in experimental groups (\*  $P < 0.050$  compared to LPS)

Histological evaluation of renal tissue demonstrated a marked increase in inflammatory cells and more fibrotic tissue and collagen content in LPS group than control (Figure 6).

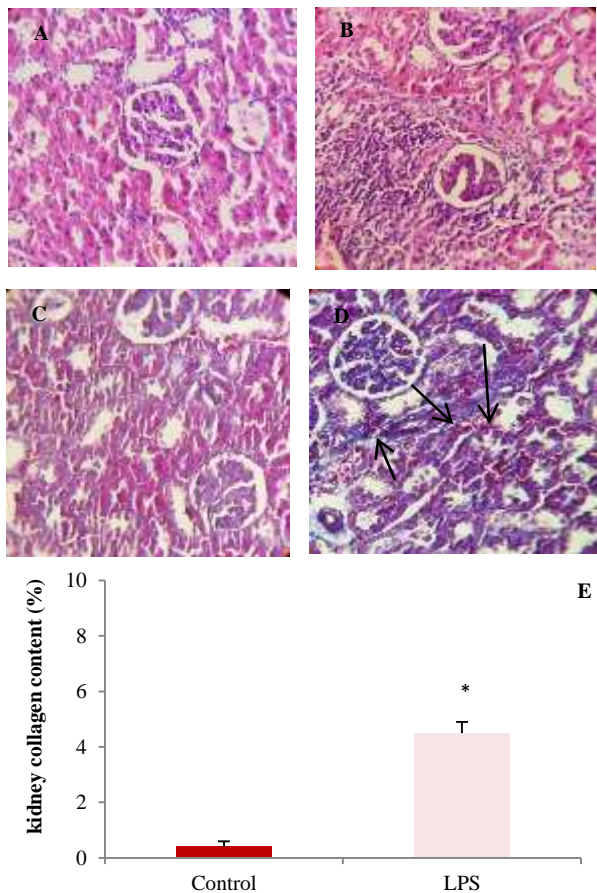
## Discussion

Our results showed that chronic low grade inflammation increased heart and kidney factors of oxidative stress, and reduced antioxidative markers. In addition, histopathological examination indicated increased inflammatory cells and collagen deposition in heart and kidney tissues.

In this study, we used subclinical LPS model. The rodent has capacity to tolerate recurrent LPS injection without lethality and this makes them as suitable model for chronic model. Study by Lew et al.,<sup>17</sup> revealed that weekly injection of 10 mg/kg LPS intraperitoneally increased serum LPS levels which is comparable to human;<sup>25,26</sup> and induced a level found in several human conditions.<sup>8</sup>

Previous studies demonstrated that LPS exacerbates hepatic, renal, and cardiac fibrosis in preexisting abnormalities. In the liver in bile duct

ligation model or after hepatic injury, fibrosis was promoted by LPS from gut microbial.<sup>27</sup>



**Figure 6.** Images A and B are hematoxylin and eosin (H&E)-stained sections demonstrating infiltration of inflammatory cells in kidneys from lipopolysaccharide (LPS)-treated rats. Masson's trichrome stained sections demonstrate collagen deposition (blue color, black arrows) in the kidneys from rats in control and LPS groups (C and D). E: Percent of collagen content in the kidneys in experimental groups (\* $P < 0.010$  compared to control)

Renal fibrosis was also promoted by TLR4 activation in urethral ligation model.<sup>28</sup> Cardiac fibrosis was also exacerbated in myocarditis.<sup>29</sup> In this study, we found that low grade inflammation induced cardiac and renal fibrosis without preexisting heart or kidney injury. Our results were in agreement with that of Lew et al. who used LPS and induced subclinical inflammation in mice.<sup>17</sup> They found that after two and four weeks of LPS administration, collagen fraction area in the left ventricle was higher compared with control group. In addition, they showed that LPS increased cardiac IL-6 and nitric oxide (NO) synthase expression. They also examined if cardiac fibroblasts are target

of LPS, and showed that incubation of heart fibroblast with LPS for 48 hours dose-dependently increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) messenger ribonucleic acid (mRNA) expression. In our study, although we did not measure inflammatory cytokine expression, however, we found that LPS administration increased serum and cardiac nitrite, serum IL-6 and TNF- $\alpha$  concentrations. IL-6 has a role in fibrosis in different organs by differentiation of fibroblasts to myofibroblasts which produces more collagen.<sup>30</sup> In addition, cardiac and renal fibroblasts increased IL-6 expression after exposure to LPS.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) and TNF- $\alpha$  are common mediators of tissue fibrosis in pathological conditions.<sup>31</sup> Since TGF- $\beta$  is not involved in cardiac fibrosis in our model,<sup>17</sup> we did not measure it. Interestingly, we showed that oxidant/antioxidant balance altered in the heart and kidney in subclinical LPS. Higher MDA concentration, lower SOD and catalase activity and total thiol concentration suggest that lipid peroxidation increased and antioxidant enzyme was suppressed after recurrent LPS. It is indicated that LPS promotes the production of inflammatory cytokines that in turn leads to excessive production of free radicals and oxidative stress. It is indicated that higher reactive oxygen species (ROS) is associated with tissue fibrosis in chronic renal failure and myocardial infarction.<sup>32</sup> In the present study, we used LPS for induction of chronic inflammation. It should be considered that in several common diseases in human such as rheumatoid arthritis (RA) and atherosclerosis, the exact mechanism for induction of chronic inflammation is different, and this is the limitation of study. On the other hand, we did not measure LPS level and some serum markers such as TGF- $\beta$  in serum and thus, we cannot compare it with human plasma level in the same condition.

## Conclusion

We concluded that conditions with chronic low grade inflammation which is common in high-fat diet, metabolic syndrome or smoking are associated with cardiac fibrosis as a primary effect which may lead to heart failure (HF) with preserved ejection fraction in chronic condition. Further studies in different models of inflammatory disease especially in chronic condition such as rheumatoid should be done to approve our findings and to find the exact mechanism of inflammation-induced fibrosis.

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### Conflict of Interests

Authors have no conflict of interests.

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