Original article

Effect of Nigella Sativa and Angiotensin converting Enzyme Inhibitor on Myocardial Fibrosis Induced by lipopolysaccharide

Hanan I. El-Kerdasy, Bodour Q. Badr Eldeen, Ali M. Ali

Department of anatomy, Benha faculty of medicine, Benha University, Egypt.

Correspondence to: Hanan I. El-Kerdasy, Department of anatomy, Benha faculty of medicine, Benha University, Egypt.

Email:

mhanan909@yahoo.com

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Abstract:

Background: Nigella sativa (NS) has anti-inflammatory and anti-oxidant activity; it exhibits a role in tissue fibrosis and wound healing. Angiotensin-converting enzyme (ACE) inhibitors improve the cardiac fibrosis. **This study aimed to** evaluate the effect of Nigella Sativa and ACE inhibitors and combination of both on myocardial fibrosis induced by lipopolysaccharide. **Material and methods:** Thirty-five male albino rats were divided into five groups. 1. Control group 2. LPS group in which rats were injected intraperitoneally (IP) with lipopolysaccharide (LPS) at a dose of (1 mg/kg/day) daily for three weeks. 3. NS treated group in which rats were IP injected with LPS then given NS oil orally at a dose of (1ml/ day) daily for three weeks 4. Captopril (CAP) treated group in which rats were IP injected with LPS then given CAP orally at a dose of (100 mg/kg/ day) daily for three weeks. 5. NS&CAP treated group in which rats were IP injected with LPS then given NS and CAP. Then the left ventricular tissues were examined for histopathological and immunohistochemical changes**. Results:** LPS induced loss of architecture of the cardiomyocytes with marked fibrosis indicated by significant increase in area % of collagen fibrosis and α-SMA immunostaining expression. NS and CAP improved these changes but the combination of both revealed marked improvement of the histopathological changes with significant decreasing in area % of collagen fibrosis and α-SMA immunostaining expression. **Conclusion:** we concluded that the combination of Nigella sativa and Angiotensin-converting enzyme inhibitor markedly improved the myocardial fibrosis induced by lipopolysaccharide.

Key words: Nigella sativa, Angiotensin-converting enzyme inhibitors, lipopolysaccharide, α-SMA immunostaining, myocardial fibrosis

Introduction

Myocardial fibrosis induced when the cardiac fibroblasts produce collagenous connective tissue. Fibrosis reduces the provider of oxygen and nourishment to the myocardium (1). Myocardial fibrosis causes structural changes that predispose to ischemia, arrhythmias and heart failure (2).

Lipopolysaccharide was found on the outer membrane of gram-negative bacteria (3). It induces a systemic and chronic low-grade myocardial inflammation. It also induces cardiomyocyte apoptosis and decreases the myocardial function (4).

LPS binds to toll-like receptors (TLRs), cell membrane receptors, of different cells, including leukocytes, endothelial cells. Cardiac myocytes also have TLRs (5). Also, LPS releases numerous cytokines such as Tumor Necrotic Factor (TNF-α), Interleukin IL-6, and C-reactive protein, and induces elevation of oxidative stress markers. It decreases the contractile function of the heart (6).

Nigella sativa (NS) is a small black seed that has been used in herbal medicine. The seed comes from a flowering plant, it can be used as anti-oxidant, antimicrobial, anticancer and antihistamine (7).

Thymoquinone is the main active ingredient of Nigella sativa. Thymoquinone has anti-inflammatory and anti-oxidant properties in the treatment of inflammatory diseases (8).It has cardio protective effects against chemical cardiotoxicity, lowers heart rate and improves endothelial function and cardiac contractility. It also has several cardiovascular effects including hypolipidemic, hypotensive, anti-platelet activities, and anti-atherogenic function (9&10).

Angiotensin converting Enzyme Inhibitors ACE was effective in treatment of inflammatory cardiovascular diseases. ACE inhibitors had a major role in the regulation of circulatory homeostasis, as it increased the prostaglandins and vascular endothelial growth factor and increased the vascular permeability and regulated inflammatory markers (11).

Captopril was one of ACE inhibitors used in treatment of hypertension, congestive heart failure and myocardial infarction. Captopril prevents the exchange of angiotensin I to angiotensin II and regulates the prostaglandins, and promotes the systemic vasodilation (12)

Recently, there was a growing interest in using natural medicinal plants compounds for treatments of several conditions including cardiovascular diseases. So, we perform this study to evaluate the effect of Nigella Sativa and Angiotensin converting Enzyme Inhibitors and the combination of both on myocardial fibrosis induced by lipopolysaccharide

Material and methods

Animals:

This experimental study was done on thirty-five male albino rats weighing 200 ± 20 g. They were housed at controlled temperatures in a 12:12 h light/dark cycle and had free access to water and diet for 2 weeks prior to the initiation of the experiment. The procedures were reviewed and approved by the research ethical committee of animal care and use of Benha faculty of medicine, Benha University. This study was done from July 2019 to October 2019 in Anatomy & Embryology Department.

Drugs:

Lipopolysaccharide (LPS) drug was purchased from Sigma (Sigma Chemical Co., USA), at a dose of 1 mg/kg/day. It was dissolved in 0.5 ml saline. Nigella Sativa oil was obtained from Cap Pharm El Captain Company, Egypt. Captopril was obtained from capoten 50 mg tablets L.L.C; SmithKline Beecham, Company Cairo, Egypt, each tablet dissolved in 5ml saline each ml contained 10 mg captopril.

Experimental design

Thirty-five rats were included in our study. After acclimatization in the lab for two weeks, the animals were randomly divided into five groups as follow:

- Group I (control group): fifteen rats were divided into 3 equal subgroups

Subgroup a: five rats were injected intraperitoneally with 0.5 ml saline, the vehicle of LPS, daily for three weeks.

Subgroup b: five rats were given Nigella Sativa oil orally at a dose of (1 ml/ day) daily via gastric tube for three weeks (13).

Subgroup c: five rats were given Captopril orally at a dose of (100mg/kg/day) daily via gastric tube for three weeks (14)

- Group II (LPS group): five rats were injected IP with LPS at a dose of (1 mg/kg/day) daily for three weeks (15).
- Group III (NS treated group):five rats were injected IP with LPS at a dose of (1 mg/kg/day) daily then given Nigella Sativa oil orally at a dose of (1 ml/ day) daily via gastric tube for three weeks (15)
- Group IV (CAP treated group): five rats were injected IP with LPS at a dose of (1 mg/kg/day) daily then given Captopril orally at a dose of (100 mg/kg/day) daily via gastric tube for three weeks (14)

- Group V(NS& CAP treated group): five rats were injected IP with LPS at a dose of (1 mg/kg/day) daily then given Nigella sativa and Captopril the same dose as in group III &IV respectively daily via gastric tube for three weeks.

After the end of the third week all living rats were sacrificed under deep anesthesia with diethyl ether. The hearts were excised by middle thoracotomy incision. The left ventricles specimens prepared for histological examination.

Histopathological examinations

Left ventricles specimens were fixed in 10% buffered formalin and embedded in paraffin then serially sections of 5 µm thickness samples were taken, then stained with hematoxylin and eosin (H&E) and Masson's trichrome stains (16&17)

Alpha smooth muscle actin Immunostaining (α-SMA)(18)

Sections of 5 µm thicknesses were dewaxed, rehydrated and washed with PBS. The sections were incubated in a humid chamber with the primary antibody anti-alfa smooth muscle actin antibody (1:500 dilution) in PBS overnight then washed and co-incubated with biotinylated secondary antibody (Dako North America, Inc, CA, USA) for one hour at room temperature. Streptavidin–biotin–peroxidase was added for 10 minutes and rinsed three times in PBS. The immunoreactivity was visualized using 3 diaminobenzidine (DAB) hydrogen peroxide. The sections were counterstained with Mayer’s hematoxylin. Semiquantitative analysis of the extension of the immunoreactivity was determined by assessing the area percentage.

α-SMA was an actin isoform that plays an important role in fibro genesis and it could be found in smooth muscles, myofibroblasts and blood vessels.

Morphometric study

The mean area percentage of collagen fibers deposition (Masson’s trichrome stain) and α-SMA immunostaining was quantified in ten images from ten non-overlapping fields of each group rats using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Statistical analysis

All data collected from the experiment was recorded and analyzed using IBM SPSS Statistics software for Windows, Version 22 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test was used to compare differences among the groups of morphometric results. In each test, the data was expressed as the mean (M) value, standard deviation (SD) and the differences were considered to be significant at $P < 0.01$.

Results

Histopathological examination:

H&E stained sections of the left ventricle from rats in the control group showed normal cardiomyocytes with acidophilic sarcoplasm, having central oval vesicular nuclei and flat nuclei of fibroblast of the connective tissue (fig 1).

H&E stained sections of the left ventricle from rats in group II (LPS group) showed loss of normal architecture and irregular cardiomyocytes with fragmentation of cardiomyocytes, hemorrhage, wide intercellular space and pyknotic nuclei(fig 2).

H&E stained sections of the left ventricle from rats in group III(NS treated group) showed mild improvement as there were normal cardiomyocytes with acidophilic sarcoplasm, having central oval vesicular nuclei, but there were wide intercellular space and pyknotic nuclei (fig 3).

H&E stained sections of the left ventricle from rats in group IV(CAP treated group) showed moderate improvement as there were normal architecture of cardiomyocytes with vesicular nuclei but there were wide intercellular space and pyknotic nuclei (fig 4).

H&E stained sections of the left ventricle from rats in group V (NS &CAP treated group) showed normal cardiomyocytes with acidophilic sarcoplasm, having central oval vesicular nuclei and flat nuclei of fibroblast of connective tissue (fig 5).

Masson's trichrome stained sections of the left ventricle from rats in the control group showed little amount of collagen fibers (fig 6). But that in group II showed marked amount of collagen fibers (fig 7). While in group III there were moderate amount of collagen fibers (fig 8). Moreover, in group IV there were mild amount of collagen fibers (fig 9). Regarding sections in group V there were little amount of collagen fibers (fig 10).

α-SMA Immunostaining:

In α-SMA immunostained sections of the left ventricle; the control group showed negative α-SMA expression in the cardiac muscle fibers (fig 11). But, that in group II showed high positive α-SMA expression in the cardiac muscle fibers (fig 12). Moreover, in group III showed moderate positive α-SMA expression (fig 13). While, group IV showed mild positive α-SMA expression (fig 14) and in group V there was negative α-SMA expression in cardiac muscle fibers (fig 15).

Morphometric results:

The mean area % and standard deviation of collagen fibers deposition was represented in (Table 1) and α-SMA immunostaining for all groups were represented in (Table 2).There was a significant increased (*P*< 0.01) in mean area % of collagen fibers deposition and α-SMA immunostaining of groups II, III &IV compared with groups I & V and there was no significant difference between the results of groups III and IV.

Table (1): Showing the mean area % and SD of collagen fibers deposition for all groups with comparison between all groups by Post Hoc LSD test.

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0.27%	20.35%	9.73%	5.44%	0.72%
SD	0.0323	1.2877	0.8542	0.5716	0.0310
Significance (sig)at P < 0.01	2,3,4	1,3,4,5	1,2,5	1,2,5	2,3,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV 5=sig. with group V

Table (2): Showing the mean area % and SD of α-SMA immunostaining for all groups with comparison between all groups by Post Hoc LSD test.

	Group I	Group II	Group III	Group IV	Group V
Mean area %	0.09%	1.81%	0.91%	0.60%	0.21%
SD	0.0385	0.2984	0.0613	0.0626	0.0517
Significance (sig)at P < 0.01	2,3,4	1,3,4,5	1,2,5	1,2,5	2,3,4

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV 5=sig. with group V

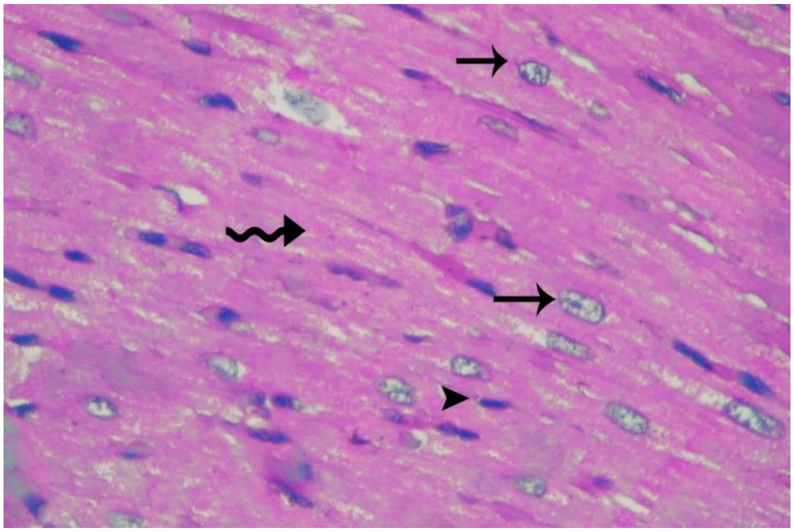

Fig 1: A photomicrograph of myocardial section of left ventricle from a rat in the control group showing normal cardiomyocytes with acidophilic sarcoplasm (wavy arrow), having central oval vesicular nuclei (arrow) and flat nuclei of fibroblast of the connective tissue (arrow head). (H&E X400)

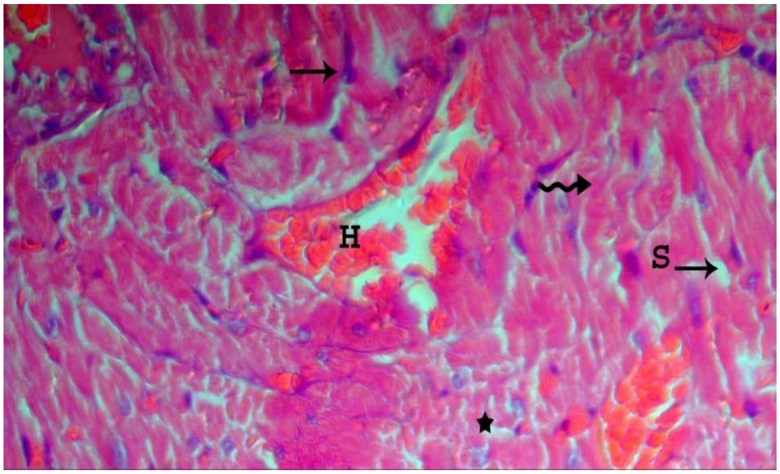

Fig 2: photomicrographs of myocardial section from a rat in the group II (LPS group) showing loss of normal architecture and irregular cardiomyocytes (wavy arrow) with fragmentation of cardiomyocytes (star), hemorrhage (H), wide intercellular space (S) and pyknotic nuclei (arrow) (H&E X400)

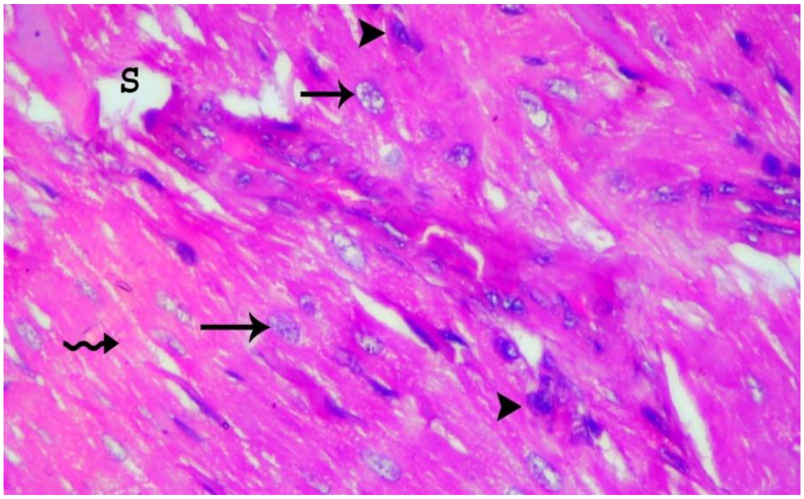

Fig 3: A photomicrograph of myocardial section from a rat in the group III (NS treated group) showing normal cardiomyocytes with acidophilic sarcoplasm (wavy arrow), having central oval vesicular nuclei (arrow) also there were wide intercellular space (S) and pyknotic nuclei (arrow head) (H&E X400)

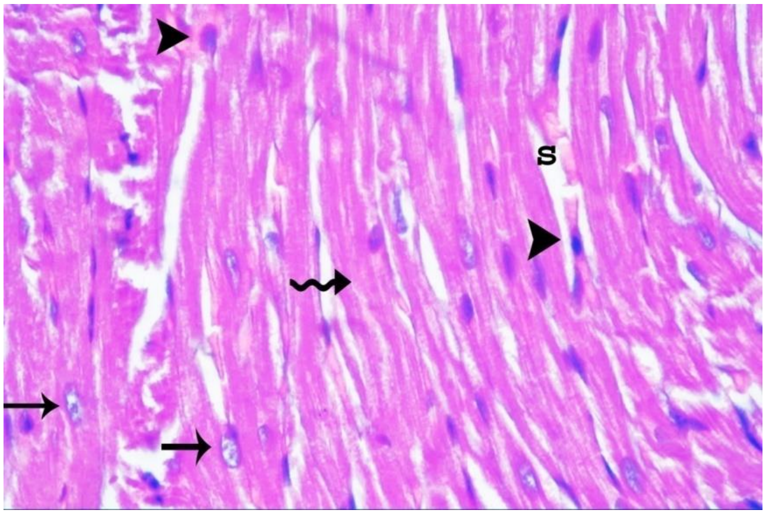

Fig 4: A photomicrograph of myocardial section from a rat in the group IV (CAP treated group) showing normal architecture of cardiomyocytes (wavy arrow) with vesicular nuclei (arrow) but there were wide intercellular space (S) and pyknotic nuclei (arrow head). (H&E X400)

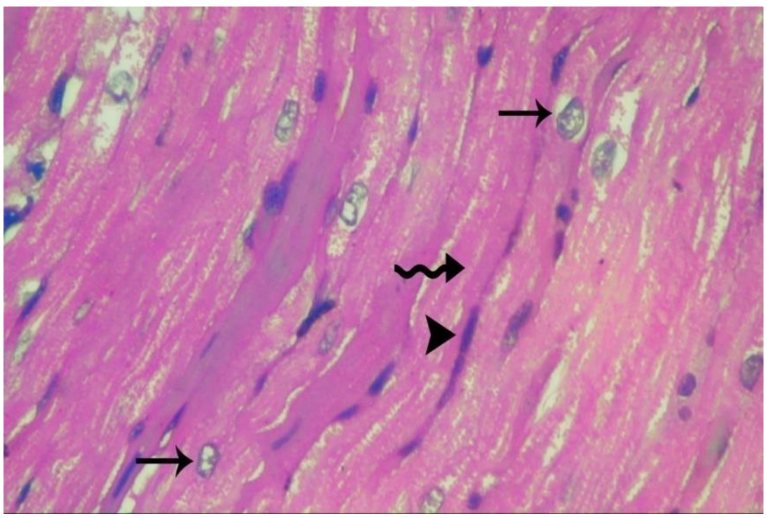

Fig 5: A photomicrograph of myocardial section from a rat in the group V (NS & CAP treated group) showing normal cardiomyocytes with acidophilic sarcoplasm (wavy arrow), having central oval vesicular nuclei (arrow) and flat nuclei of fibroblast of connective tissue (arrow head). (H&E X400)

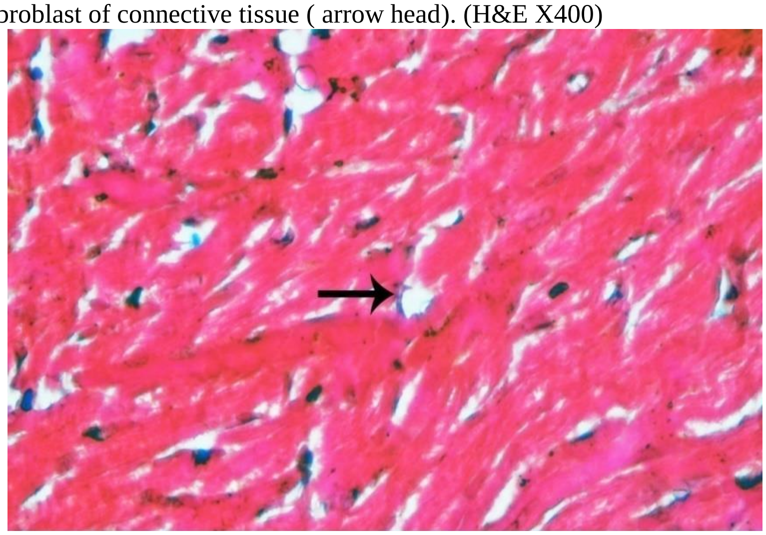

Fig 6: A photomicrograph of myocardial section from a rat in the control group showing little amount of collagen fibers (arrow) (Masson trichrome X 400)

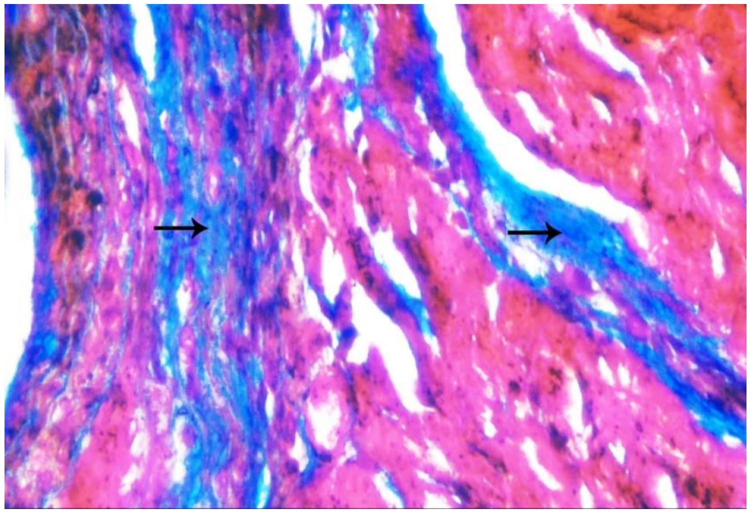

Fig 7: A photomicrograph of myocardial section from a rat in the group II (LPS group) showing marked large amount of collagen fibers (arrow) (Masson trichrome X 400)

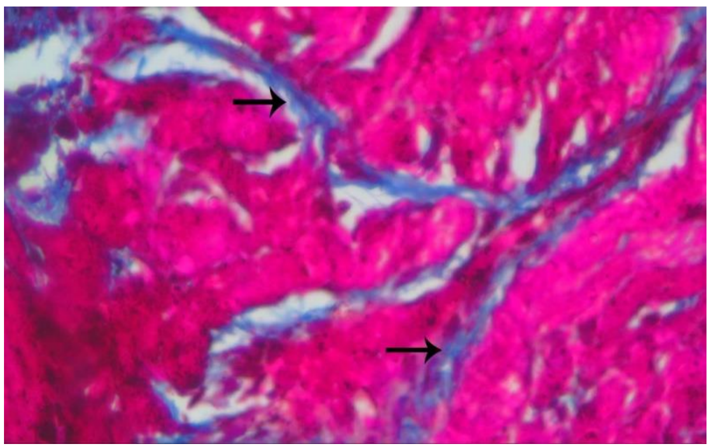

Fig 8: A photomicrograph of myocardial section from a rat in the group III (NS treated group) showing moderate amount of collagen fibers (arrow) (Masson trichrome X 400)

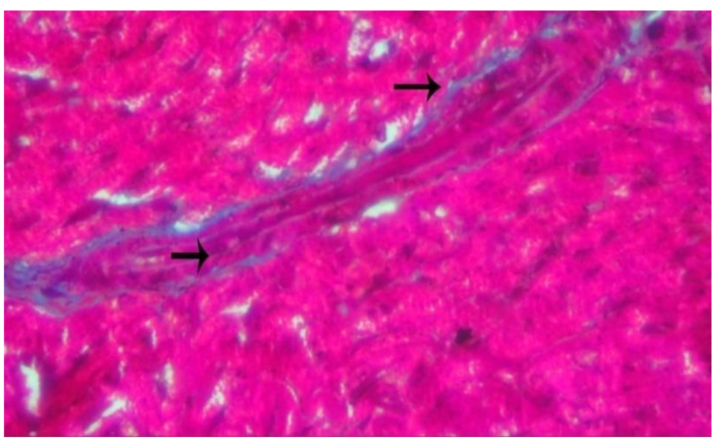

Fig 9: A photomicrograph of myocardial section from a rat in the group IV (CAP treated group) showing mild amount of collagen fibers (arrow) (Masson trichrome X 400)

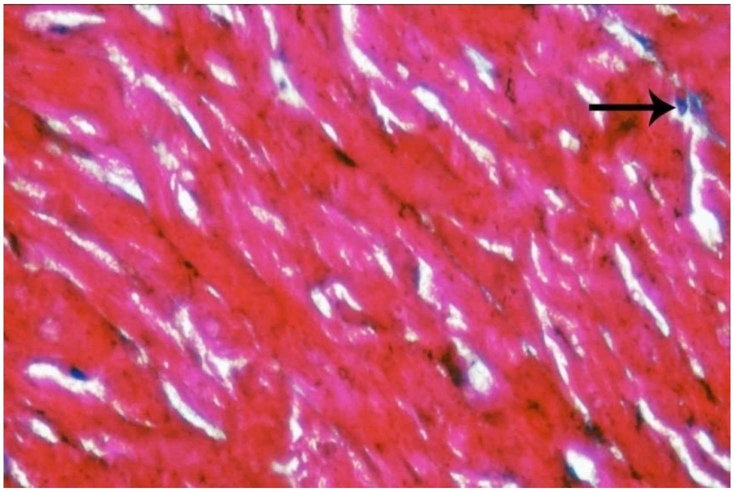

Fig 10: A photomicrograph of myocardial section from a rat in the group V (NS & CAP treated group) showing little amount of collagen fibers (arrow) (Masson trichrome X 400)

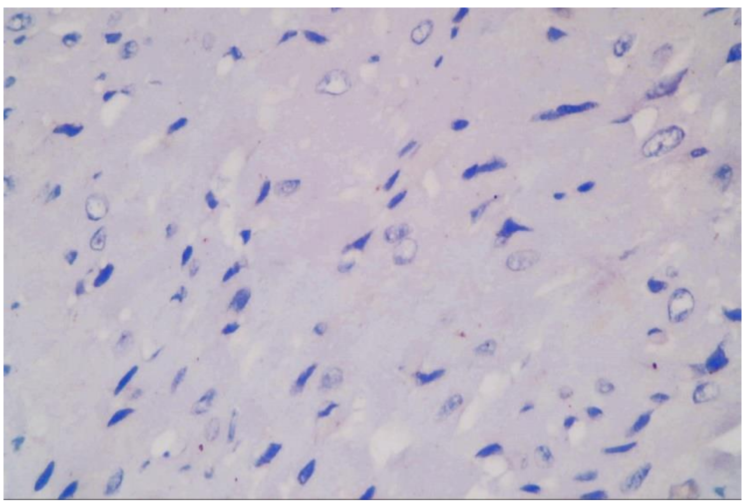

Fig 11: A photomicrograph of myocardial section from a rat in the control group showing negative α-SMA expression in the cardiac muscle fibers (α-SMA immunostaining X400)

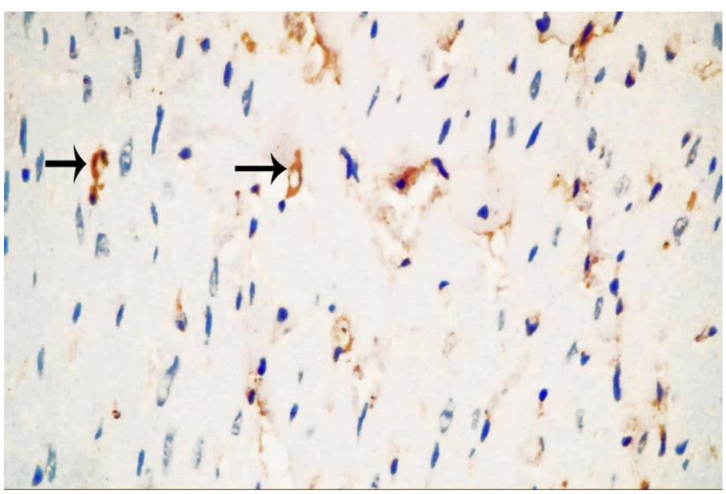

Fig 12: A photomicrograph of myocardial section from a rat in the group II (LPS group) showing high positive α-SMA expression brown stain (arrow) in the cardiac muscle fibers. (α-SMA immunostaining X400)

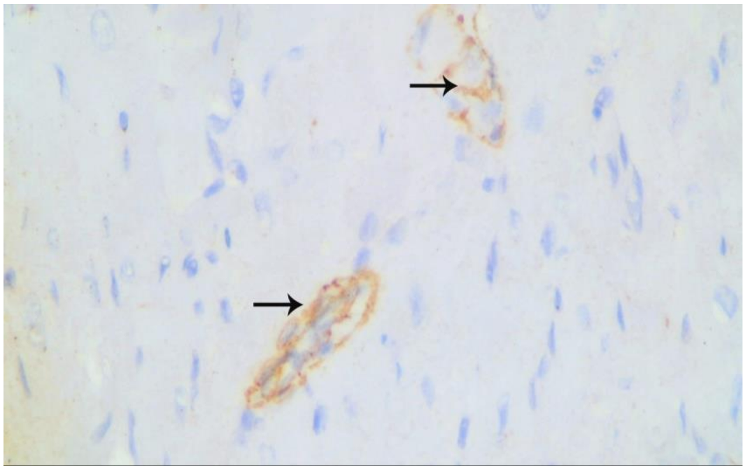

Fig 13: A photomicrograph of myocardial section from a rat in the group III (NS treated group) showing moderate positive α-SMA expression brown stain (arrow) in the cardiac muscle fibers (α-SMA immunostaining X400)

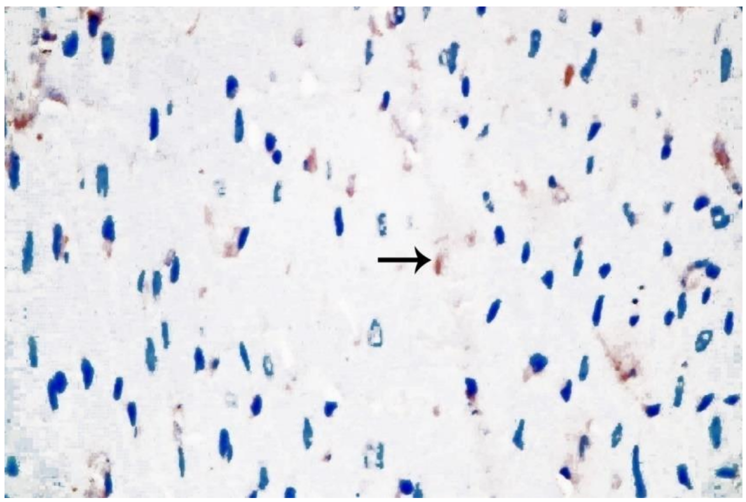

Fig 14: A photomicrograph of myocardial section from a rat in the group IV (CAP treated group) showing mild positive α-SMA expression brown stain (arrow) in the cardiac muscle fibers (α-SMA immunostaining X400)

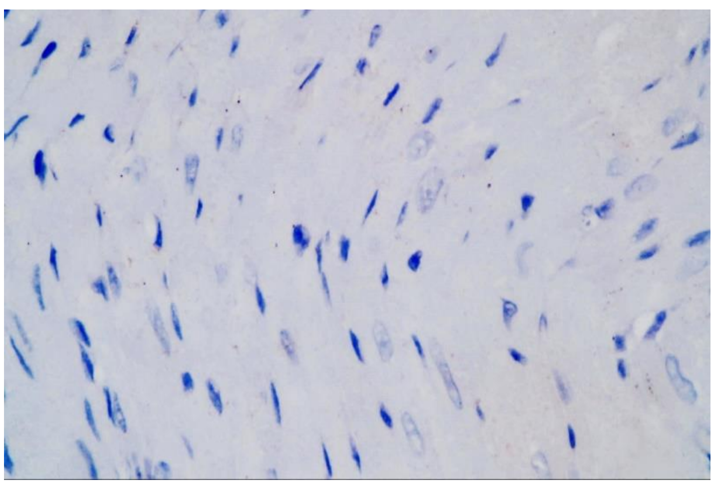

Fig 15: A photomicrograph of myocardial section from a rat in the group V (NS & CAP treated group) showing negative α-SMA expression in the cardiac muscle fibers (α-SMA immunostaining X400)

Discussion

The effect of recurrent exposure to subclinical LPS change ventricular structure and function, as low levels of LPS activate cardiac apoptosis through the renin angiotensin system that induces cardiac fibrosis and depresses cardiac contractility (19).

Thymoquinone (TQ) the active component of Nigella sativa has numerous cardiovascular effects; inotropic, antihypertensive, hypolipidemic, and has a great beneficial value in cardiac toxicity and prevention of ischemia and reperfusion injury. TQ exerts these effects mostly through its antioxidant and anti-inflammatory properties (20, 21). Therefore, it is used in the treatment of inflammatory diseases and has valuable effect on cardiovascular diseases (22).

ACE inhibitors have cardiac antiproliferative and antifibrotic effects as it inhibits the conversion of angiotensin I to Ang II, kinin hydrolysis. It also increases the concentrations of N-acetyl-seryl-aspartyl-lysyl-proline in plasma and tissue (11).

The present study designed to evaluate the effect of Nigella Sativa and Angiotensin-converting enzyme inhibitor and combination of both in myocardial fibrosis induced by lipopolysaccharide.

In the present study, all results revealed that a picture of myocardial degeneration and fibrosis was detected in LPS group in the form of loss of normal architecture and irregular, fragmented cardiomyocytes, hemorrhage, significantly increased collagen fibers...etc. These results were in agreement with a study which (15) reported that the administration of LPS induced myocardial and per-vascular fibrosis and increased inflammatory markers and cardiac oxidative stress. In the same line, other authors reported that recurrent exposure to subclinical LPS has main adverse long-term cardiac fibrosis (23). Similarly, confirmed our results and found that chronic low-grade inflammation induced cardiac fibrosis which may lead to heart failure (4). Other studies reported that LPS caused metabolic endotoxemia with low-grade inflammation, and insulin resistance that enlarged cardiovascular risk. LPS was an influential trigger for the intrinsic immune system response, through binding to the Toll-like receptor 4 and its co receptors (24). Also, it was found that LPS- induced acute kidney injury, LPS-stimulated endotoxemia via alterations in the expression level of Sequestosome-1 p62 protein (25).

NS promotes coronary angiogenesis through increasing the expression of antigens and growth factors (26). Several mechanisms were suggested in the literature to explain the hypotensive effect of NS; through its action as a calcium channel blocker, its effect on increasing heart rate and cardiac contractility, or its activation of muscarinic receptors on blood vessels (27).

The current study demonstrated that NS administration showed mild improvement of LPS induced myocardial fibrosis in rats. Similarly, it was concluded that administration of nigella sativa improved myocardial fibrosis in LPS inflammation-induced fibrosis model (13). Also, it was found that the treatment of rats with thymoquinone had protected the heart muscle against hypothyroidism- induced histopathological and immunohistochemical changes (22). Moreover, a study reported the effect of thymoquinone in attenuates liver fibrosis and demonstrated that TQ may be a potential candidate for the therapy of hepatic fibrosis (28). These results could be explained by that NS acts through alteration of oxidative/antioxidative balance raising antioxidative enzymes. Other authors reported that thymoquinone, dose-dependently, inhibited the LPS-induced inflammatory mediators and prostaglandin E2 production (29).

Furthermore, the current study reviled that captopril administration could moderately improve LPS induced cardiac fibrosis. This was in accordance with the study which found that administration of captopril reduced the severity of the pathological changes induced by lipopolysaccharide (11). Other authors recorded that captopril was mildly ameliorates the collagen and α-SMA expression in diabetic rats (14). This could be explained by the rule of ACE inhibitor in decreasing elimination of bradykinin, a peptide which is inotropic and increases coronary blood flow (30).

In addition, other studies stated that, ACE inhibitors exert a beneficial rule in treatment of hypertension and heart failure, not only by the conversion of angiotensin I to Ang II, but also by reducing the degradation of bradykinin, which enhances coronary blood flow and cardiac contractility in ischemic situation (31). Bradykinin also induces the release of vasoactive modulator such as nitric oxide and prostaglandin (32). ACE inhibitors caused vasodilatation and decreased aldosterone secretion due to decreased in plasma angiotensin II. The decrease in aldosterone secretion leads to increase in serum potassium and sodium levels, with fluid loss (33).

Finally, this study demonstrated marked improvement of myocardial fibrosis induced by LPS in administration combination of Nigella Sativa and captopril. Other study indicated the effect of TQ on ischemia-reperfusion injury in isolated rat heart as it significantly improved cardiac function by suppression of oxidative stress, and apoptosis, as well as reduced the cardiac lactate dehydrogenase (LDH) and creatine kinase levels (34). Also (35) reported that captopril improved the left ventricular function and reduced cardiac vasculitis, necrosis, hypertrophy and ischemia.

Other authors studied the antifibrotic effect of captopril and enalapril on paraquat induced lung fibrosis in rats and their histopathological examination revealed that both captopril and enalapril improved pulmonary fibrosis (36).

Conclusion:

This study concluded that the combination of Nigella Sativa and Angiotensin-converting enzyme inhibitor markedly improved the myocardial fibrosis induced by lipopolysaccharide.

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