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The Cardioprotective Effects of Aminoguanidine on Lipopolysaccharide Induced Inflammation in Rats

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Abstract

Myocardial dysfunction, a major component of sepsis-induced multiorgan failure, contributes to the production of massive amounts of pro-inflammatory cytokines. Nitric oxide (NO) is known to act as a precursor of free radicals in inflammation. This research was conducted to assess the effect of aminoguanidine (AG) on lipopolysaccharide (LPS)-induced heart injury. 50 male rats were categorized into five groups ($n = 10$): (1) control, (2) LPS, (3) LPS-AG50, (4) LPS-AG100, and (5) LPS-AG150. LPS (1 mg/kg) was injected for 5 weeks, and AG (50, 100 and 150 mg/kg) was injected 30 min prior to LPS administration. All drugs were injected intraperitoneally. LPS-evolved cardiovascular toxicity was indicated by the augmentation in the level of nitric oxide (NO) metabolites, interleukin (IL)-6 and malondialdehyde (MDA), as well as reduced contents of total thiol groups, catalase (CAT), and superoxide dismutase (SOD) activity in serum, heart, and aortic tissues. In AG treated groups, noxious effects of LPS were not observed in the serum and harvested tissues. AG reduced MDA, NO metabolites, and IL- 6 and increased total thiol, CAT, and SOD activity in the heart, aorta and serum. As an inhibitor of inducible NO synthase (iNOS), AG further reduced LPS-induced oxidative stress and inflammation, hence considered as cardioprotective.

Keywords Lipopolysaccharide · Heart · Aminoguanidine · Oxidative stress · Inflammation

Introduction

Heart failure, a last communal path of numerous cardiovascular diseases, is one of the primary causes of death worldwide. It encompasses sustained pressure overload (such as hypertension), myocardial ischemia or infarction, volume overload (such as valvular heart disorders), and inherited or acquired cardiomyopathies. Inflammation and oxidative damage appear to be important causes involved in the progression of heart failure [1].

In the present research, lipopolysaccharide (LPS) was utilized to induce systemic inflammation. LPS is a key component of the outer membrane of Gram-negative bacteria. The immune system is continually exposed to low amounts of LPS in low-grade bacterial infections. The recognition and signaling responses of LPS are crucial to eliminating the attacking pathogens [2]. The LPS-induced activation of macrophages, prompts the generation of bioactive lipids, reactive oxygen species (ROS) [3], and particularly, inflammatory cytokines. Although LPS response is essential for fighting and eliminating bacterial infections, it also mediates deleterious host reactions [4]. Inflammation has a significant role in triggering and developing numerous cardiovascular diseases such as atherosclerosis [5]. LPS receptors or Toll-like receptors (TLRs) are expressed in cardiomyocytes which have a main role in cardiovascular diseases such as myocardial dysfunction. TLR-4 and TLR-2 knockout mice showed better cardiac function

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following sepsis compared to wild-type ones [6]. Activating TLRs causes the nuclear translocation of nuclear factor κB (NF-κB), enhancing cytokines formation and expressing the adhesion molecules [7]. These events could be very important in cardiomyopathies. LPS can trigger extra formation of inflammatory mediators, causing lethal systemic diseases, including septic shock and tissue damages. Inflammation can inflict oxidative damage through producing oxidative agents such as superoxide anion and nitric oxide (NO).As an extremely reactive free radical, NO plays multiple roles in physiological and pathological procedures [8]. Inducible NO synthase (iNOS) yields great amounts of NO which reacts with superoxide and generates peroxynitrite, mainly in the immune cells. Numerous extracellular stimuli including cytokines, LPS, and oxidative stress activate NF-κB as an inducible transcription factor [9]. Via TLRs, these agents stimulate innate immunity and prompt systemic inflammation. TLRs are observed in cells with or without immune function like endothelial cells and cardiomyocytes [6]. Endotoxin-induced oxidative stress status was characterized by altering antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and excessive ROS accumulation in myocardial tissue; this causes cellular injury by impairing vital macromolecules and changing membrane fluidity and mitochondrial function [10].

Moreover, it has been reported that anti-inflammatory and antioxidant compounds are able to apply protective effects on organs [11]. In this context, iNOS inhibitors have been revealed to have important cellular protective effects on reducing lipid peroxidation and total nitrite level; also, a previous study reported their anti-inflammatory, antioxidant and anti-apoptotic properties [12]. Aminoguanidine (AG) is an iNOS inhibitor with a wide range of physiological properties, including suppression of inflammation, neuro-protective function, and inhibition of oxidative and nitrosative stress [13]. Moreover, AG has been found to suppress DNA binding NF-κB that eventually inhibits the formation of inflammatory cytokines [14]. There are several reports on the advantageous properties of AG on inflammatory responses and oxidative stress; however, the impact of this inhibitor on cardiovascular dysfunction resulting from LPS is yet to be elucidated.

In this study, we evaluated the effect of AG, a well-known iNOS inhibitor, on serum, aorta and heart inflammatory markers, and oxidative stress status regarding empirically-induced inflammation in male rats.

Materials and Methods

Animals

Male Wistar rats (240 ± 10 g) were purchased from the local laboratory animal center at Mashhad University of Medical Sciences. The animals were kept in standard cages and maintained under ethical conditions (humidity of 56 ± 1%, temperature 23 ± 1 °C, and periodic 12 h light/dark condition). Food and water were accessible with no restrictions. The Ethics Committee of Animal Research approved experimental processes (Ethical code: IR.MUMS.fm.REC.1397.35).

Chemicals and Animal Groups

Prior to injection, LPS and AG (Sigma-Aldrich Chemical Co) were dissolved in sterile saline. The rats were randomly classified into five groups ($n = 10$) according to the subsequent protocol: (1) control group, which received saline in place of both LPS and AG, (2) LPS group, receiving LPS (1 mg/kg/day; i.p.) and 1 ml/kg saline in place of AG over 4 weeks (3–5) LPS-AG 50, LPS-AG 100, and LPS-AG 150 groups, administered with a daily injection of LPS (1 mg/kg/day; i.p.) over 4 weeks and 50, 100, and 150 mg/kg of AG dissolved in saline (i.p.) 30 min prior to the administration of LPS. Drug dosage and treatment duration were selected according to the previous similar studies [15–18].

Biochemical Assessments

Finally, the animals were deeply anesthetized by overdose (1.6 g/kg; i.p.) of urethane, their blood specimens were collected, and their heart and aorta were separated. The phosphate buffer solution (pH 7.4) was utilized to homogenize the tissues. The centrifugation of homogenates was performed at 1500 rpm for 10 min to measure the malondialdehyde (MDA), total concentration of thiol, superoxide dismutase (SOD) and catalase (CAT) activities, NO metabolites concentration, and interleukin (IL)-6 level.

Oxidative Stress Criteria

MDA was determined as a lipid peroxidation biomarker. MDA measurement technique was reported earlier. In general, one ml of the sample solution was combined with 2 ml of thiobarbituric acid (TBA) + trichloroacetic acid (TCA) + hydrochloric acid (HCl) solution and placed in boiling water bath for 45 min. Finally, its absorbance (A) of the solution was recorded at 535 nm following its centrifugation.

MDA concentration (C) was calculated using the following formula: $C\ (M) = A/1.65 \times 10^5$ [19, 20].

The total thiol contents were determined in the tissue homogenates and serum by use of a technique introduced by Ellman [21]. In general, 50 µl of the supernatant or serum of all samples and 1 ml of tris-ethylenediaminetetraacetic acid (EDTA) buffer were combined, and the absorbance was recorded at 412 nm against tris–EDTA buffer alone labeled A1. Afterwards, 20 µl of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) solution was poured into A1; the sample

absorbance was then recorded for the second time following 15 min, labeled A2. The absorbance of DTNB was utilized as blank. An equation was utilized to compute the total thiol concentration:

$$\text{Total thiol concentration}: (\text{mM}) = (\text{A2} - \text{A1} - \text{B}) \times 1.07/0.05 \times 13.6$$

SOD activity was determined on the basis of Madesh and Balasurbamanian protocol. The technique is based on the formation of SOD via the auto-oxidation of pyrogallol and dependent on the inhibition of 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) to formazan. The reaction was stopped with dimethyl sulfoxide (DMSO). In brief, the sample supernatant was added into the plate wells (96 wells). Following 5 min, the DMSO was poured, and the plate was observed via a microplate reader at 570 nm. One unit of SOD was defined as the level of protein required for the inhibition of 50% decrease in MTT [22].

To measure CAT activity, 100 µl of H_2O_2 was combined with phosphate buffer (pH = 7) and employed to prepare the solution C. 650 µl of phosphate buffer (pH = 7) was used as the blank solution. Measurements were performed via buffer C and sample homogenates. The absorption reduction was recorded using a spectrophotometer at 240 nm. The conversion of H_2O_2 into H_2O and O_2 in 1 min under standard condition was considered as the enzyme response velocity [23].

Evaluation of NO Metabolites and IL-6 Concentration

Levels of NO (NO_2/NO_3) metabolites were measured using the Griess reaction method. In brief, after adding 100 µl supernatant or serum to the Griess reagent, contents were transferred to a 96-well flat-bottomed microplate; absorbance was further read at 520 nm using a microplate reader, and the final values were calculated from standard calibration plots [12]. The Ebioscience ELISA kit (Ebioscience Co, San Diego, CA, USA) and the instructions provided by the manufacturer were used to measure IL-6 levels in the tissues. The concentrations were calculated through comparing the absorbance of the samples recorded in the microplate reader (Biotek, USA) with a standard curve determined in the same measurement.

Statistical Analysis

The analysis was conducted via SPSS 20.0 statistical package (IBM SPSS Inc., USA). Data were reported as means ± SEM. One-way ANOVA and Tukey's post hoc tests were employed to evaluate the biochemical data. Differences were considered statistically significant if $P < 0.05$.

Results

Thiol and MDA Concentrations

Compared to the control animals, the LPS-treated animals showed higher levels of MDA and lower levels of thiol in heart and aorta tissues as well as in serum ($P < 0.001$). Administration of the two higher dosages, containing 100 and 150 mg/kg of AG, reduced heart, aorta, and serum levels of MDA compared with the LPS group ($P < 0.001$). Moreover, the two higher dosages of AG increased the total concentration of thiol in the heart, aorta, and serum compared with LPS group ($P < 0.05$–< 0.001) (Fig. 1).

SOD and CAT Activity

The results further showed that administrating the LPS reduced SOD activity in heart, aorta, and serum ($P < 0.001$). This impact of LPS was reduced by the two maximum dosages of AG in heart and serum ($P < 0.05$–< 0.001). Furthermore, in the aortic tissue, all three dosages of AG significantly increased SOD activity ($P < 0.05$–< 0.001).

The administration of LPS reduced heart, aorta, and serum CAT activity compared to the control group ($P < 0.001$). Pretreatment with 150 mg/kg of AG enhanced CAT activity in the heart and aortic tissues ($P < 0.001$). Also, pretreatment by all dosages of AG increased the activity of CAT in the serum ($P < 0.05$–< 0.001) (Fig. 2).

NO Metabolites

The heart, aorta, and serum levels of NO in LPS group were significantly increased as compared with the control group ($P < 0.001$). Pretreatment with all dosages of AG (50, 100 and 150 mg/kg) reduced NO levels in the heart ($P < 0.05$–< 0.001).

Administrating two higher dosages of AG (100 and 150 mg/kg) reduced NO in the aorta and serum ($P < 0.001$) (Fig. 3).

IL-6

The heart, aorta, and serum levels of IL-6 in LPS group were significantly increased in comparison to the control group ($P < 0.001$). Pretreatment with all dosages of AG (50, 100 and 150 mg/kg) reduced IL-6 levels in the heart

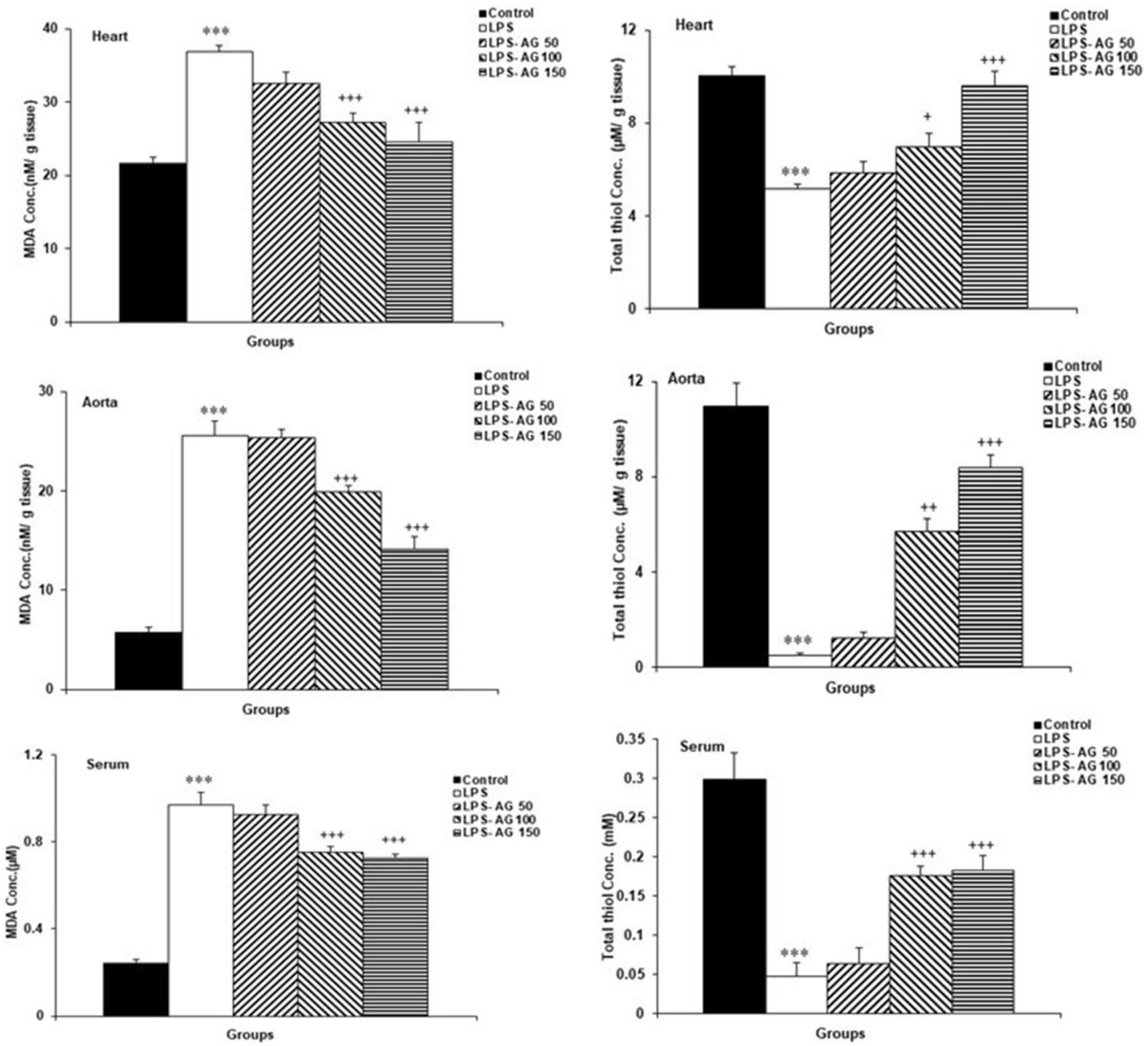

Fig. 1 Comparison of the MDA and total thiol concentrations in the heart, aorta tissues and serum. Results are presented as mean ± SEM ($n = 10$ in each group). ***$P < 0.001$ compared to the control group. $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to the LPS group

and serum ($P < 0.05$–< 0.001); however, in the aorta, pretreatment with two higher dosages of AG (100 and 150 mg/kg) reduced the levels of IL-6 ($P < 0.001$) (Fig. 3).

Discussion

The current research investigated the potential protective effect of AG, as iNOS inhibitor, on cardiovascular dysfunction caused by LPS in rats. Acute inflammation causes a general reaction in the organism, leading to hypoglycemia, anorexia, loss of body weight, and changes in the serum levels of numerous plasma proteins formed by the hepatocytes [24]. Inflammation-evolved injury has been reported in myriad organs such as the brain, liver, heart, lung, pancreas, kidney, gonads, and intestines [25]. Numerous stimuli regarding diverse origins such as bacterial infection, endotoxemia, and sterile tissue damage (surgical traumas, burns, and ischemic necrosis) are able to activate the host inflammatory responses [24]. These conditions can be replicated via several empirical treatments. Specifically, intra-peritoneal injection of LPS is usually employed to reproduce systemic inflammation; moreover, subcutaneous injection of turpentine leads to the creation of sterile abscesses and peripheral tissue damage [26]. LPS was used on animals as an experimental model to investigate the

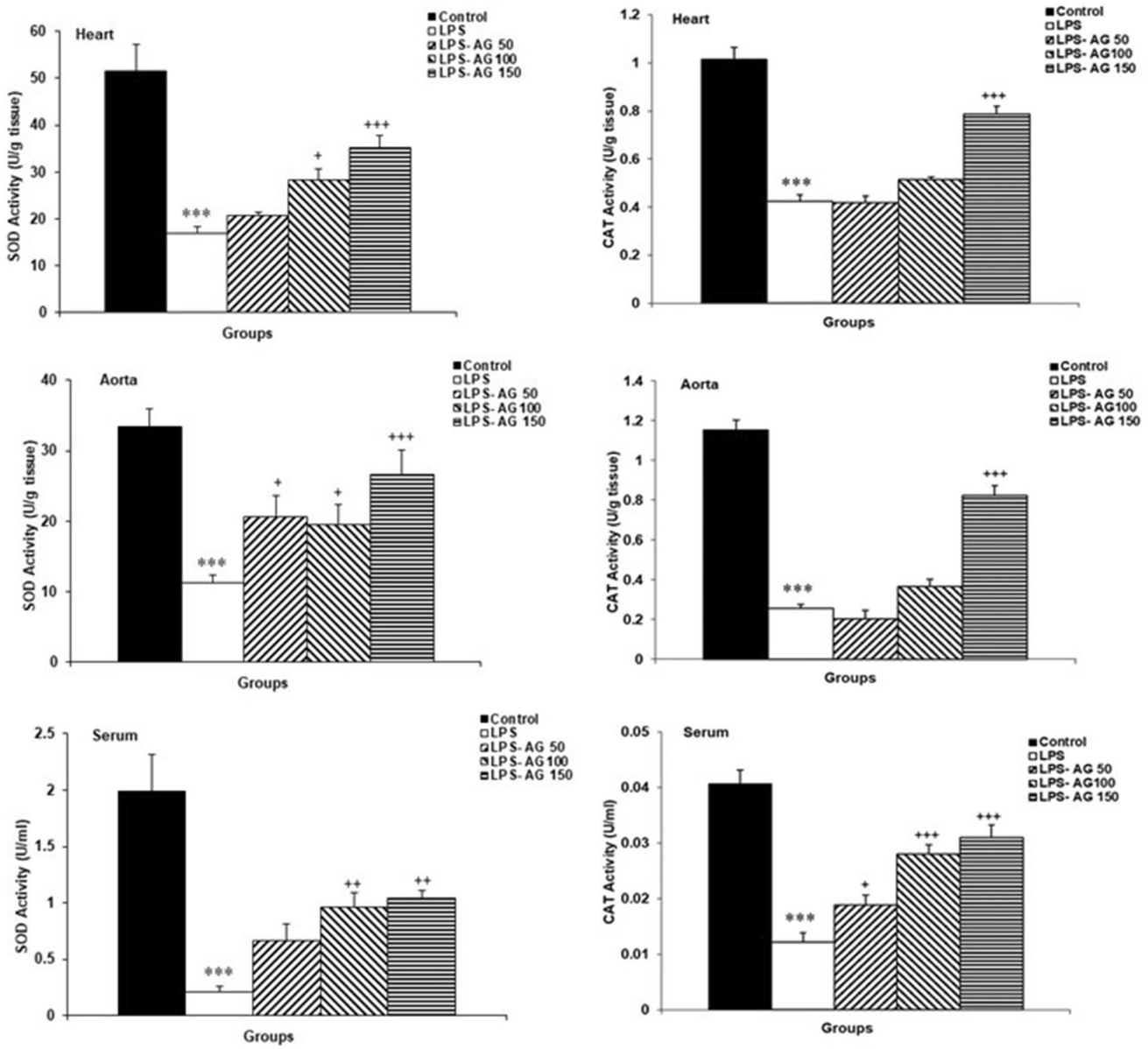

Fig. 2 Comparison of the SOD and CAT activities in the heart, aorta tissues and serum. Results are shown as mean ± SEM ($n = 10$ in each group). ***$P < 0.001$ compared to the control group. $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to the LPS group

mechanism of endotoxin-induced tissue damage because it infiltrated inflammatory cells [27]. In the present study, LPS was shown to cause inflammation through increasing IL-6 levels in the aorta, heart, and serum. Numerous cytokines, mainly IL-1, TNF-α, and IL-6, are involved in the regulation of these phenomena; however, their specific roles have not yet been fully elucidated in various aspects of inflammation and in response to different stimuli [28]. IL-1 and IL-6 are formed in response to various inflammatory stimuli [29]. The experiments with neutralizing antibodies have shown that inhibiting the activity of each of these cytokines reduces several systemic responses to inflammation [30]. LPS is characterized by increased production of ROS and lipid peroxidation products, including peroxides and superoxide anions and their by-products such as MDA [31]. The current available data indicated that the injection of LPS increased NO metabolites and MDA while reduced the total thiol concentrations as well as SOD and CAT activity. In another study, LPS injection was followed by an oxidative stress status in rat [3]. NO is a free radical molecule with different physiological and pathological functions under manifold conditions [32]. This highly reactive mediator is produced from L-arginine in the presence of three different isoforms of NO synthase (NOS) including endothelial (eNOS), neuronal

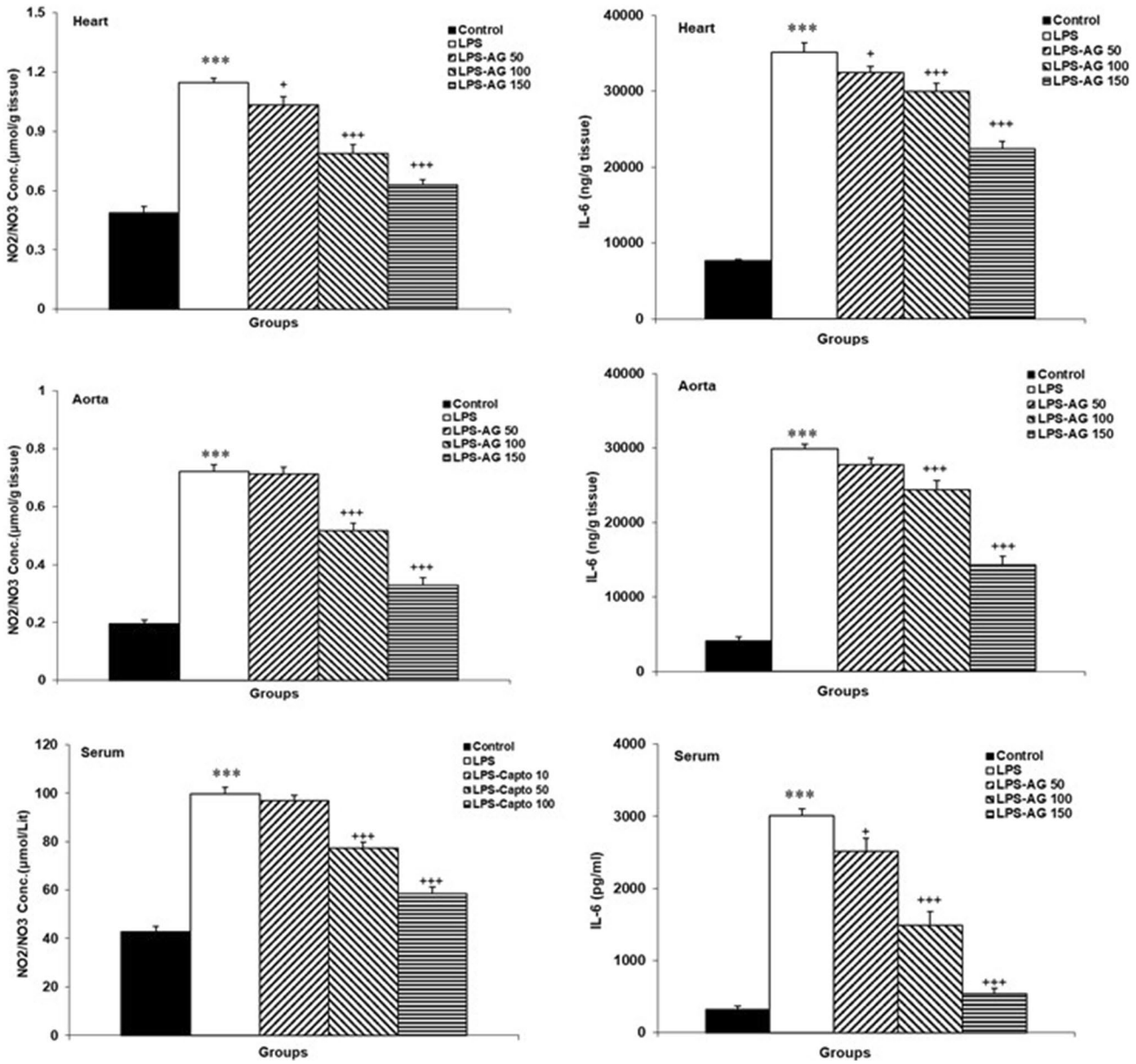

Fig. 3 Comparison of the level of nitric oxide metabolites and IL-6 in the heart, aorta tissues and serum. Results are shown as mean ± SEM ($n = 10$ in each group). $^{***}P < 0.001$ compared to the control group. $^{+}P < 0.05$ and $^{+++}P < 0.001$ compared to the LPS group

(nNOS) and inducible NOS (iNOS) [14, 33]. iNOS is an isoform expressed in the immune system and involved in the development of cell death through forming NO and pro-inflammatory cytokines containing IL-1β and TNF-α [14, 34]. In our study, it was observed that iNOS blockade with AG might inhibit LPS-induced inflammatory and oxidative stress responses, possibly ameliorating the cardiovascular function in rats.

The beneficial effects of AG seem to be owing to the inhibition of lipid peroxidation and NO synthesis. AG was further reported to have antioxidant, anti-inflammatory, and anti-apoptotic properties [33]. An overproduced level of TNF-α and other cytokines was reported to have a pivotal role in inducing iNOS and developing cell toxicity [35]. Moreover, iNOS inhibition by AG suppressed TNF-α production in an animal model of ischemia–reperfusion [36]. The results of these studies are in accordance with the current research where AG injection reduced the content of IL-6 in LPS-AG groups compared to LPS group.

Also, the preventive effects of AG against cellular damage were observed to be due to its antioxidant properties [37]. In this case, AG improved the cisplatin-caused nephrotoxicity via antioxidant effects [38]. Furthermore, AG reinforced the endogenous antioxidant defense system and restored the

radiation-induced lung toxicity in rats [39]. Peroxynitrite, as a product of NO, was able to attenuate cellular antioxidants such as total thiol groups, glutathione (GSH), and antioxidant enzymes [40, 41]. This deleterious agent is produced as a combination product of superoxide with NO and acts as an active molecule. Reduced peroxynitrite was reported to have a role regarding the preventive effects of AG against cyclophosphamide-induced renal injury [42]. Similar to these studies, in the present research, AG improved LPS-caused oxidative stress through reducing the MDA and NO levels and enhancing the antioxidant defense systems, including SOD, CAT, and thiols in serum, heart and aorta. Considering these data, it is proposed that improving the impacts of AG on cardiovascular function in LPS-treated rats may be facilitated over its development effect against oxidative stress injury followed by the inflammation caused by LPS in serum, heart, and aortic tissues of rats.

Conclusion

The results of the present study showed that AG improved cardiovascular dysfunction due to the inflammation caused by LPS in rats. The cardiovascular improving effect of AG can be attributed to the reduction in the inflammation and oxidative stress response although further research is required to delineate the precise mechanism(s). Evaluating the expression of the related genes and proteins involved in the assumed action mechanism of AG or even the histopathological investigations are highly recommended.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval All experiments were conducted in accordance with the Animal Experimentation Ethics Committee of MUMS (Approval No. IR.MUMS.fm.REC.1397.35).

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