Saudi Pharmaceutical Journal 30 (2022) 1405–1417

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

Original article

Preclinical study for the ameliorating effect of L-ascorbic acid for the oxidative stress of chronic administration of organic nitrates on myocardial tissue in high sucrose/fat rat model

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ARTICLE INFO

Article history:
Received 20 December 2021
Accepted 13 July 2022
Available online 19 July 2022

Keywords:
Methemoglobin
Organic nitrates
Oxidative stress
L-ascorbic acid

ABSTRACT

Background: The therapeutic activity of Glyceryl trinitrate (GTN) is mainly regulated by liberating nitric oxide (NO) and reactive nitrogen species (RNS). During this biotransformation, oxidative stress and lipid peroxidation inside the red blood cells (RBCs) occur. Hemoglobin tightly binds to NO forming methemoglobin altering the erythrocytic antioxidant defense system.
Aim: The principal objective of our research is to show the ameliorating effect of L-ascorbic acid for the deleterious effects of chronic administration of nitrovasodilator drugs used in cardiovascular diseases such as oxidative stresses and tolerance.
Method: We studied some biochemical parameters for the oxidative stress using groups of high sucrose/-fat (HSF) diet Wistar male rats chronically orally administered different concentrations of Isosorbide-5-mononitrate (ISMN) 0.3 mg/kg, 0.6 mg/kg and 1.2 mg/kg. Afterwards, we evaluated the role of L-ascorbic acid against these biochemical changes in cardiac tissues.
Results: Chronic treatment with organic nitrates caused elevated serum levels of lipid peroxidation, hemoglobin derivatives as methemoglobin and carboxyhemoglobin, rate of hemoglobin autoxidation, the cellular levels of the pro-inflammatory cytokines marker (*NF-κB*) and apoptosis markers (*caspase-3*) in the myocardium muscles in a dose-dependent manner. Meanwhile, such exposure caused a decline in the enzymatic effect of SOD, GSH and CAT accompanied by a decrease in the level of mitochondrial oxidative stress marker (*nrf2*) in the myocardium muscles and a decrease in the serum iron and total iron-binding capacity (TIBC) in a dose-dependent manner. Concomitant treatment with L-ascorbic acid significantly diminished these changes for all examined parameters.
Conclusion: Chronic administration of organic nitrates leads to the alteration of the level of oxidative stress factors in the myocardium tissue due to the generation of reactive oxygen species. Using L-ascorbic acid can effectively ameliorate such intoxication to overcome nitrate tolerance.

1. Introduction

Hypertension, congestive heart failure, angina pectoris, ischemic heart disease and cerebrovascular disease are the worldwide main reason for deaths and aliment adjusted life years (Steven et al., 2018, Gori, 2020). ISMN is an organic nitrate vasodilator that represents a promising therapeutic option for both treating and preventing such cardiovascular diseases. On the molecular level, GTNs, including the organic nitroglycerins, act as pro-drugs for nitric oxide which is a highly effective vasodilator drug. Bioactivation of nitrate occurs when inert nitrite is converted

Abbreviations: ALDH-2, Aldehyde Dehydrogenase–2; CAT, Catalase Activity; GTN, Glyceryl trinitrate; HSF, High Sucrose/Fat; NO, Nitric Oxide; RNS, Reactive Nitrogen Species; SOD, Superoxide Dismutase; TIBC, Total Iron Binding Capacity; XO, Xanthine Oxidase.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.jsps.2022.07.001

to NO by mitochondrial aldehyde dehydrogenase–2 (Signorelli et al., 2020, da Silva et al., 2021). NO plays an important role in relaxing the vascular smooth muscle and increasing blood flow to the myocardium (Mullane et al., 2020). GTNs are recommended for the treatment of stable coronary artery disease that is still symptomatic despite treatment with aspirin, beta-adrenergic receptor blockers or ACE inhibitors (Kim et al., 2020).

The use of GTN is widely limited by its chronic administration procedure due to the rapid emergence of tolerance and/or endothelial dysfunction. In most cases, tolerance is explained by the increased intracellular oxidative stress. The anti-ischemic effect of GTNs is rapidly lost with chronic administration of low doses. This phenomenon is explained by the reactions of RNS; NO, peroxynitrite and Nitric dioxide, with lipids and other biomolecules under increased intracellular oxidative stress (Daiber and Münzel, 2015, Knuuti et al., 2019). The undesirable and poorly tolerated hemodynamic side-effects of GTNs such as headache and orthostatic hypotension can often occur owing to systemic vasodilatation (Ivy, 2019).

Treatment with GTNs raises the cellular level of generated reactive oxygen species (ROS) within the vascular tissue. SOD, CAT and GSH are cellular defense mechanisms providing protection against the damage effect of free radicals and ROS (Pearson and Butler, 2021). GTNs chronic administration leads to biotransformation into NO in the reticulocytes of the vascular tissues which in turn causes oxidative stress, SOD inhibition, depletion of GSH pool and decreased bioavailability of NO (Awad et al., 2018).

Tolerance due to chronic administration of GTNs, reduction of its clinical response, leads to the urgent need of increasing the GTNs dosages in order to maintain the vasodilating effect (Tauzin et al., 2018). The molecular mechanism of this tolerance includes that GTNs interferes with purine metabolism and drain intracellular ATP and GTP. GTNs block xanthine oxidase (XO), a rate-limiting enzyme for the metabolic bioactivation of GTNs into NO. L-ascorbic acid prevented XO blockage which results in an increased NO production from GTN (Axton et al., 2018, Marini et al., 2022). Nitrate tolerance is handled by generating a 12-h nitrate-free interval. Although this strategy can manage nitrate tolerance, it results in an increased risk of cardiovascular problems during these free periods, especially in the early light phase (Axton et al., 2018).

NO metabolism mainly occurs in the RBCs (Graham et al., 2021, Vaz-Salvador et al., 2022). Besides, NO bioactivity is preserved by RBCs. Metabolism of GTNs leads to the formation of inorganic nitrates that can oxidize hemoglobin into methemoglobin (Awad et al., 2018).

Organic nitrates cause oxidation of hemoglobin (Hb) resulting in methemoglobin (MetHb) formation and O_2 liberation. This autoxidation reaction of oxyhemoglobin is super rapid (Tai et al., 2020). Such elevated MetHb content is a dose-dependent on the organic nitrates concentrations (Hathazi et al., 2018). MetHb production catalysis binding of oxygen to normal hemoglobin, so less oxygen will be available at the tissue level (Wang et al., 2013).

Iron is present in the ferric state (Fe^{3+}) in methemoglobin, so it cannot bind to oxygen. This procedure is irreversible resulting in NO oxidation to nitrate and NO resistance. Subsequently, serious hypertension, lipid peroxidation, and vascular injury are followed. Both cellular oxidative stress and inflammatory tissue injury can be occurred due to the released heme and globin as a result of the catabolism of NO and/or oxyhemoglobin and methemoglobin (Fidanzio et al., 2021, Cao et al., 2021). GTN tolerance is accompanied by raising the cellular level of free radical-induced lipid peroxidation (Wang et al., 2021, Meegan et al., 2021). Nitrate tolerance has been considered to be a limitation of nitrate therapy. The effectiveness and benignity of nitrate therapy could be dramatically improved (two to three times) upon concomitant administration of antioxidants which prevents the need for nitrate-free intervals (Marini et al., 2022, Knuuti et al., 2020).

Ascorbic acid is a potent electron donor water-soluble antioxidant. It efficiently preserves the body functions against oxidative stress and is a cofactor in numerous essential enzymatic reactions (Jurt et al., 2001). Ascorbic acid enhances endothelial vasodilation and prevents endothelial dysfunction (Cartaya et al., 2019, Doseděl et al., 2021). Antioxidant agents are responsible to convert methemoglobin back to hemoglobin (Plotnick et al., 2017, Cartaya et al., 2019).

2. Material and methods

2.1. Drugs and chemicals

ISMN was obtained from Sigma-Aldrich Chemical Co., Merck KGaA, Darmstadt, Germany. It was freshly dissolved in double-distilled water (0.3, 0.6 and 1.2 mg/kg). L-ascorbic acid was obtained from El-Gomhoria Co., Cairo, Egypt (20 mg/kg). It was dissolved in double-distilled water. All other chemicals and reagents were analytical grade and were purchased from El-Nasr Co., Cairo, Egypt.

2.2. Animal treatment

As previously described in Kottaisamy et al. (2021), Forty-eight 4-week-old male Wistar rats (experimental animal house, Cairo University, Egypt) fed with HSF diet (body weight, 192.19 ± 8.12 g) placed in conventional cages (3 rats/cage) were divided into eight groups in a random manner. The HSF diet consisted of a 79% normal diet, 10% sucrose, 5% lard, 5% cholesterol, and 1% lithocholic acid. They were kept for two weeks before the experiments started in rooms with controlled temperature (23 ± 2 °C) and humidity (50 ± 5%) and a 12/12 h light/dark cycle starting at 6:00 am. Both their weight and their caloric intake were recorded weekly. The methods got approval by the ethical research project committee in the University of Tabuk (UT-44-10-2019) and all steps were performed in accordance with its relevant guidelines and regulations. The ethical committee in the Faculty of Pharmacy, University of Mansoura, Mansoura, Egypt (2019–62), approves all animal facilities and animal protocols. All animals were fed with HSF diet for twelve weeks. ISMN was dissolved in water and given orally at three different doses (0.3 mg/kg, 0.6 mg/kg and 1.2 mg/kg body weight). These range of doses has been used previously for a wide range of oral administered doses of ISMN for different conditions of heart diseases in the rat model (Li et al., 2018, Arnaud et al., 2017, Tai et al., 2020). In this study, we studied the oxidative stress effect after the chronic administration of this wide range of doses. An oral doses of L-ascorbic acid was dissolved in water and given orally (20 mg/kg body weight). Such dose was previously reported to have a cardioprotective effect against chronic administration of doxorubicin for doxorubicin-induced cardiotoxicity in the rats when they were used per oral route (Buttros et al., 2009).

2.3. Animals were divided into seven main groups as follows (Fig. 1)

Group (1): Control group which fed with HSF diet only. Water and food were given freely.

Group (2): L-ascorbic acid group which was given L-ascorbic acid 20 mg/ kg body weight orally once daily for twelve weeks.

Group (3) was given ISMN 0.3 mg/kg orally once daily for twelve weeks.

Group (4) was given ISMN 0.3 mg/kg followed by an oral dose of L-ascorbic acid.

Group (5) was given ISMN 0.6 mg/kg orally.

Group (6) was given ISMN 0.6 mg/kg followed by an oral dose of L-ascorbic acid.

Group (7) was given ISMN 1.2 mg/kg orally.

Group (8) was given ISMN 1.2 mg/kg followed by an oral dose of L-ascorbic acid.

Animals were sacrificed by cervical dislocation under light ether anesthesia.

2.4. Blood sample collection

Blood samples were drained from each rat in each group and centrifuged for isolation of the serum. Samples were stored at –70 °C.

The following investigations were done:

2.5. Hematological parameters

Methemoglobin, Carboxyhemoglobin, oxygen saturation, and oxygen content were measured using (Arnaud et al., 2017, Li et al., 2018).

Briefly, Methemoglobin is measured spectrophotometrically at 540 nm after complete transformation into Cyanmethemoglobin using potassium cyanide (Li et al., 2018. Carboxyhemoglobin was assayed spectrophotometrically with automated multi-wavelength method (Kozlova et al., 2020). A double-beam Shimadzu model UV-1900i UV–Vis Spectrophotometer (1.0 cm quartz cells) was used for all absorbance measurements.

Oxygen saturation and oxygen content were measured using Radiometer OSM3; Hemoximeter adjusted for rat blood.

2.6. Cardiac biomarkers

2.6.1. Serum lactate dehydrogenase

The activity of the serum lactate dehydrogenase was measured using a commercial kit (Sangong Biotech, Shanghai, China) according to the instruction of the manufacturer. Briefly, 10 µl of serum was mixed with a working reagent containing Tris buffer (80 mM; pH 7.4), pyruvate (1.6 mM), NaCl (200 mM) and NADH (240 mM), mixed well and incubated at 37 °C for 1 min and measured the change in absorbance per minute for 3 min duration at 340 nm.

2.6.2. Serum creatinine kinase-MB

Serum creatinine kinase-MB activity was estimated using a commercial kit (Sangong Biotech, Shanghai, China) according to the instruction of the manufacturer. Briefly, 40 µl of serum was mixed with 1 ml of a working reagent. The contents were mixed thoroughly and incubated at 37 °C for 100 s and the change in absorbance per minute was recorded spectrophotometrically at 340 nm for 5 min.

2.7. Oxidative stress parameters

2.7.1. Determination of SOD activity

The activity of SOD was also measured spectrophotometrically at the same wavelength (540 nm) as described by Boriskin et al. (2019). Briefly, the blood serum was centrifuged in for 10 min at 6000 rpm. Afterwards, we added phosphate buffer (pH 7.4) to the supernatant with ration 3:1. Then, we centrifuged at 5000 rpm for 15 min, precipitated the hemoglobin using of 2:1 chloroform: methanol solution with a ratio of 2:1 for 10 min, centrifuged the reaction mixture, diluted with phosphate buffer 20 times and incubated the mixture for 10 min with 57 µm nitro blue tetrazolium, 98.5 µm NAD·N and 16 µm fenasintrasalud in a 0.5 M phosphate buffer with EDTA (pH 8.3) at a temperature of 25 °C in aerobic conditions. The activity of SOD was calculated by the formula (Huo et al., 2021):

$$\text{Enzymatic Activity} = \frac{\textit{Pecentage of inhibition of HBT reaction}\%}{100\% - \textit{Percentage of inhibition of HBT reduction reaction}\%}$$

2.7.2. Determination of CAT activity

It was done using the standard method of Boriskin et al. (2019). Briefly, we stated ca reaction of 0.1 ml of blood serum to 2 ml of 0.03% hydrogen peroxide solution and leave for 10 min. Afterwards, we stopped the reaction with 1 ml of 4% ammonium molybdenum. The Color intensity was measured spectrophotometrically at a wavelength of 410 nm.

CAT is calculated by the formula [Rosa et al., 2021]:

$$\text{CAT} = \frac{\textit{Optical density of the blank} - \textit{Optical density of the sample}}{\textit{Volume of the sample} * \textit{time of incubation} * 1.6 * 100,000}$$

2.7.3. Determination of malondialdehyde (MDA)

The amount of MDA was measured as previously mentioned in Hamdan et al (Kumar and Gill, 2018). Briefly, we added trichloroacetic acid to the serum in order to precipitate all proteins. Then, we added thiobarbituric acid in order to form thiobarbituric acid. Then, we measured the color intensity at a wavelength of 532 nm.

2.7.4. Determination of NO level

The serum NO concentrations were measured indirectly spectrophotometrically in a two-step process. Initially, we determined the total serum NO for both nitrate and nitrite concentrations using the previously mentioned method (Csonka et al., 2015). Briefly, we added modified Griess's reagent (1% sulphanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid) to the serum for 10 min till developing of a deep purple azo mixture and the absorbance was measured at 546 nm. Then, we subtracted the directly measured serum nitrite from the measured total nitrite nitrate concentrations to calculate the serum nitrate concentrations.

2.7.5. Determination of GSH-Px activity in plasma

We measured the serum activity of GSH-Px also spectrophotometrically at 412 nm as described by Han et al. (2021). Briefly, we added 171 mM of K_2HPO_4/KH_2PO_4, 4.28 mM of NaN_3, 2.14 mM of EDTA, 6 mM of reduced GSH, 0.9 mM of NADPH, and 2 U./mL of glutathione reductase. We added 50 µl of reaction mixture containing 5 mg of 5,5′, dithiobisnitrobenzoic acid in 5 ml assay buffer stored on ice to form a complex.

2.7.6. Determination of serum iron and TIBC

Serum iron and TIBC were measured using immunoassay studies which were carried out using semi-auto analyzer Beckman Coulter AU480.

2.8. Real-Time polymerase chain reaction

We homogenized 100 mg frozen myocardium of each rat in each group of rats in 1 ml of TRIzol® (Thermo Fisher Scientific, Inc., USA). We performed all the processes for total RNA extraction according to the manufacturer's protocol. Total RNA concentration was adjusted spectrophotometrically at a wavelength of 260 nm using nuclease-free water. Real-Time was performed using a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., USA) as directed in the manufacturer's protocol.

2.9. Determination of gene expressions of nrf2, NF-κB and caspase-3

All genetic expressions of the following genes; *nrf2*, *NF-κB* and *caspase-3* were assayed as described previously (Hamdan et al., 2019). We used the sequences of the primers as shown in Table 1. The primers designed using PrimerQuest for spanning exon junctions' mode.

2.9.1. Western blotting

Total protein was extracted from cardiac lysates in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). After incubation at 4 °C for 1 h, the cell suspension was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was collected. Protein content in the supernatant was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. An equal amount of protein (50 μg) was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes using turbo *trans*-blot apparatus (BD Bioscience, USA). The membrane was blocked with 5% BSA in TBST for 1 h at room temperature. It was further washed three times with TBST for 10 min each. The membrane was incubated at 4 °C overnight in 5% BSA in TBST containing primary antibodies (Santa Cruz Biotechnology, USA) to one of the following: Nrf2 (1:1000), NF-κB (1:1000), caspase-3 (1:800) and anti-β-actin (1:1000). After washing with TBST, the membrane was incubated with peroxidase-conjugated corresponding secondary antibodies for 1 h at room temperature. After washing, images were captured on films, which were placed in Pierce ECL Western Blotting Substrate.

2.9.2. Histopathological evaluation

Directly after dissection, the heart was taken out, cleaned and fixed in 10% neutral buffered formalin solution for the preparation of histopathological slides. After fixation, tissues were dehydrated in different series of ethanol, cleared in xylene and embedded in paraffin wax. The solid sections were prepared at 5 mm thickness using a microtome, stained with haematoxylin-eosin (H&E)/ Masson's trichrome. The sections were examined under a light microscope and photographs were taken.

2.10. Statistical analysis

All results were shown as mean ± standard deviation (SD). We used a one-way analysis of variance (ANOVA). It was preceded by Tukey's post hoc test using GraphPad Prism® software version 5. For all analysis, the level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of continuous administration of GTNs on the hematological parameters

A significant increase in methemoglobin and carboxyhemoglobin, after continuous exposure to ISMN to rats ($p < 0.05$) in a concentration-dependent manner compared to the control group (Fig. 2A, B). Meanwhile, continuous administration of ISMN to rats leads to a decrease in oxygen saturation, and oxygen content, hemoglobin content ($p < 0.05$) in a concentration-dependent manner (Fig. 2C, D). Concomitant administration of the L-ascorbic acid leads to a decrease in the increased levels of methemoglobin and carboxyhemoglobin and to restoration of the decreased level of the increased levels of the hemoglobin content, oxygen saturation, and oxygen content for the ISMN treated groups.

3.2. Effect of continuous exposure to organic nitrates on the oxidative stress parameters

Continuous exposure of rats to ISMN leads to a significant increase in the level of the MDA activity ($p < 0.05$) in a concentration-dependent manner (Fig. 3A). Meanwhile, continuous exposure to ISMN leads to the reduction of the activity of all of the following; SOD, CAT, GSH and NO content level ($p < 0.05$) in a concentration-dependent manner (Fig. 3B). Concomitant administration of L-ascorbic acid leads to restoration the increased level of MDA and to ameliorating the decreased levels of SOD, CAT, GSH and NO content for the ISMN treated group (Fig. 3).

3.3. Effect of continuous administration of organic nitrates on the iron content, total iron-binding capacity and inorganic phosphorus content

Continuous administration of organic nitrates to rats leads to a reduction of both the serum iron level and TIBC ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the decreased level of the serum iron and the TIBC in the rats (Fig. 4A, B).

3.4. Effect of continuous administration of organic nitrates on the cardiac biomarkers (lactate dehydrogenase and CK-MB)

Continuous administration of organic nitrates to rats resulted in an increase of lactate dehydrogenase and CK-MB in a concentration-dependent manner ($p < 0.05$) to normal rats. Concomitant administration of L-ascorbic acid leads to restoration the increased level of the lactate dehydrogenase and the CK-MB in the rats (Fig. 5 A and B).

3.5. Effect of continuous exposure to organic nitrates on the mitochondrial oxidative stress marker (nrf2), the pro-inflammatory cytokines marker (NF-κB) and apoptosis markers (caspase-3) concentrations in the myocardium muscles

Continuous exposure to organic nitrates caused a significant reduction of the genetic expressions of both *nrf2* mRNA and the protein NRF2 as well in the myocardium tissues in a concentration-dependent manner. However, L-ascorbic acid restored the normal level of *nrf2* and NRF2 in the myocardium tissue (Fig. 6A). On the same way, continuous exposure of rats to organic nitrates led to a significant increase in the genetic expressions of nuclear factor *NF-κB* mRNA and protein in the myocardium tissue in a concentration-dependent manner (Fig. 6B).

Table 1
Primer sequence of used primers for RT-PCR.

	Gene name	Gene ID	Forward primer	Reverse primer	Product size (bp)
1	*nrf2*	NM_031789	5′-CACATCCAGACAGACACCAGT-3′	5′-CTACAAATGGGAATGTCTCTGC-3′	121
2	*NF-κB*,	NM_001276711	5′- ACGATCTGTTTCCCCTCATCT -3′	5′- TGCTTCTCTCCCCAGGAATA -3′	150
3	*caspase-3*	NM_012922	5′- GTTAACACGAGTGAGGATGTG-3′	5′- GTTACCAGGGCTGCCTTCTC-3′	446
4	GAPDH	XM_039107008	5′- GCCAAAAGGGTCATCATCTCCGC -3′	5′- GGATGACCTGCCCACAGCCTTG -3′	319

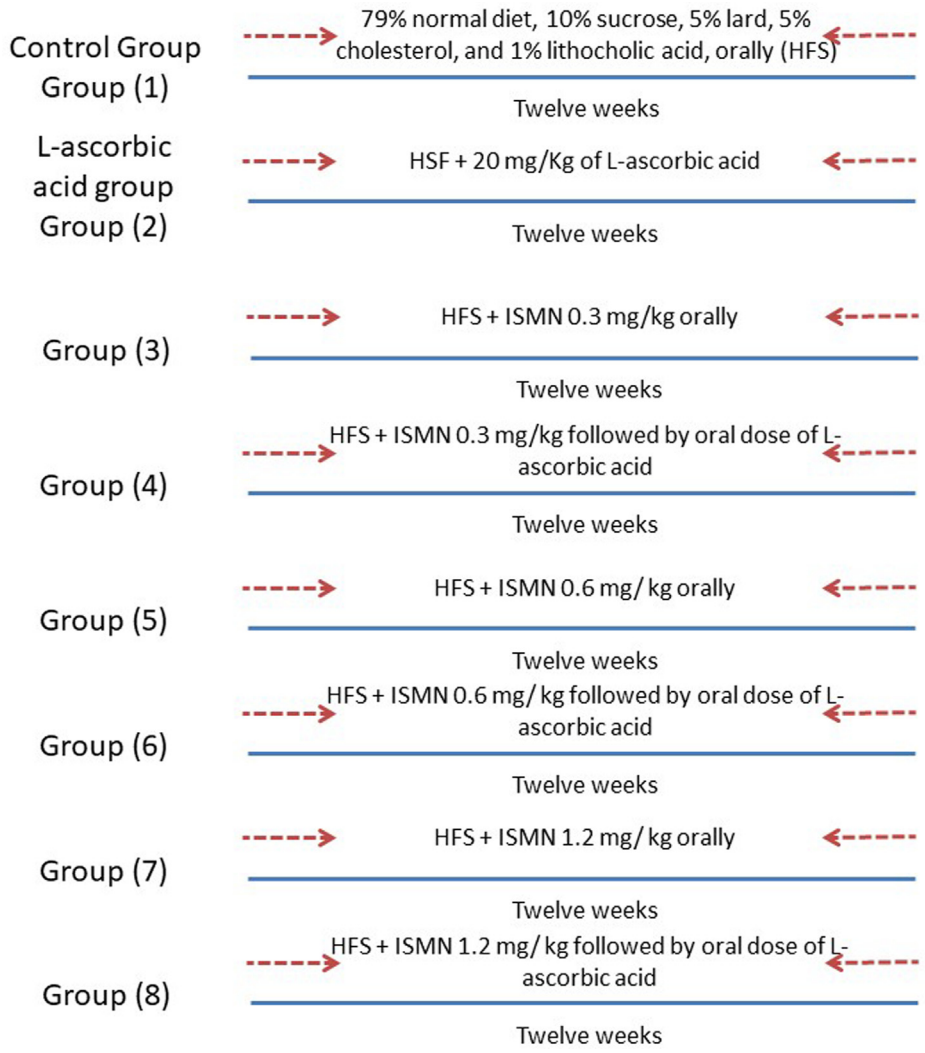

Fig. 1. Schematic representation of dosage regimen of the different studied groups. Control group received HSF diet only for twelve weeks (GPI), L-ascorbic acid group received 20 mg/kg body weight of L-ascorbic acid with HSF diet for twelve weeks (GPII), ISMN 0.3 mg group received 0.3 mg/kg of ISMN with HSF diet (GPIII), ISMN 0.3 mg + L-ascorbic acid group received 0.3 mg/kg of ISMN along with oral dose of 20 mg/kg of L-ascorbic acid with HSF diet for 12 weeks (GPIV), ISMN 0.6 mg group received 0.6 mg/kg of ISMN with HSF diet (GPV), ISMN 0.6 mg + L-ascorbic acid group received 0.6 mg/kg of ISMN along with oral dose of 20 mg/kg of L-ascorbic acid with HSF diet for 12 weeks (GPVI), ISMN 1.2 mg group received 1.2 mg/kg of ISMN with HSF diet (GPVII) and ISMN 1.2 mg + L-ascorbic acid group received 1.2 mg/kg of ISMN along with oral dose of 20 mg/kg of L-ascorbic acid with HSF diet for 12 weeks (GPVIII).

Besides, continuous exposure of rats to organic nitrates led to a significant increase in the genetic expressions of apoptotic marker *caspase-3* mRNA and protein in the myocardium tissue in a concentration-dependent manner (Fig. 6C). However, L-ascorbic acid restored the increased levels of both mRNA and proteins of NF-κB and caspase-3 in the myocardium tissue to their original levels.

3.6. Effect of continuous exposure to organic nitrates on the myocardium tissues

Continuous exposure to the HSF diet and 20 mg/kg body weight of L-ascorbic acid has no effect on the normal cardiac tissues (Fig. 7A1, 7A2, 7B1 and 7B2) and has no fibrosis (7C1 and 7C2). Exposure to 1.2 mg/kg body weight of ISMN leads to a moderate Zenker's necrosis (thin black arrows) (6A3 and 6B3) accompanied by mononuclear inflammatory cells aggregation (green head arrows), cytoplasmic vacuoles (yellow arrows) (7A3), with mild degeneration of myofibrils (thick black arrows) (7B3) and mild intermuscular fibrosis (7C3). The group treated with ISMN (1.2 mg/kg) followed by L-ascorbic acid (7A4 and 7B4) showed mild recovery of myocardial lesions with normal myofibril structures (7B4), but some remained mild necrosis (black arrow) (7A4) and a gradual decrease in intermuscular fibrosis (7C4).

4. Discussion

The act of NO is multifaced. It can be as a messenger for either a pro-oxidant or an antioxidant in biological systems (Han et al., 2021). All organic nitrates undergo biotransformation releasing NO (Lu et al., 2019). Chronic exposure to nitrates induces oxidative stress and exerts disastrous pathophysiological effects including oxidative stress, inflammation, autoimmune disease and cancer (Meera et al., 2020). This may be directly due to the potential liability of mediating DNA damage or indirectly through the production of RNS free radicals. NO has a short half-life and is widely formed through the vascular endothelium, resulting in direct relief of the smooth muscles of the vascular tissues (Lu et al., 2019). Serum levels of both Nitrates and Nitrites were used to estimate the level of NO

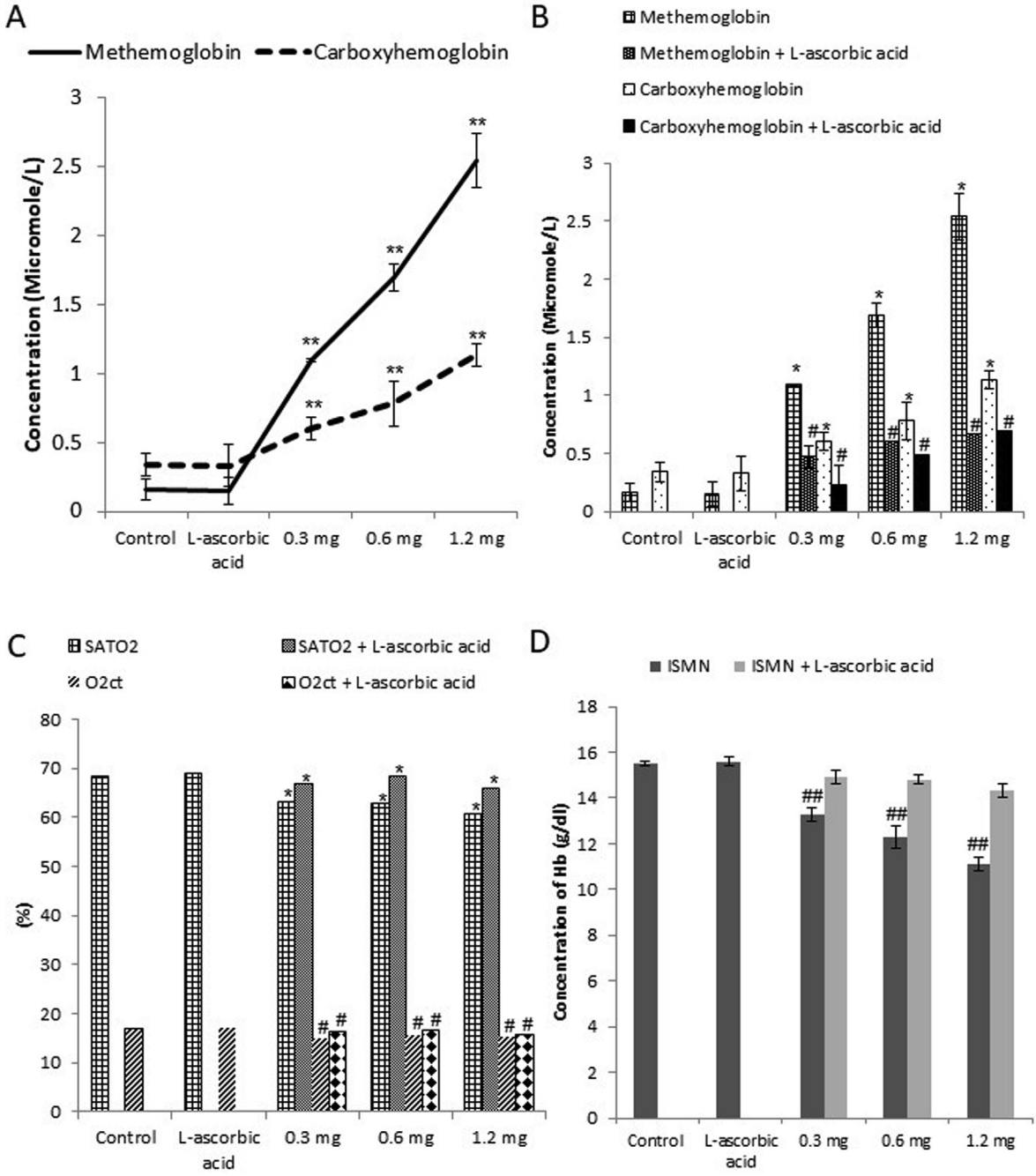

Fig. 2. Effect of continuous administration of GTNs on the hematological parameters. Data represented as means ± SD. n = 5–6. *: Significant decrease compared with control groups at $p < 0.05$. **: Significant increase compared with control groups at $p < 0.05$. #: Significant decrease compared with ISMN groups of the same dose at $p < 0.05$. ##: Significant increase compared with ISMN groups of the same dose at $p < 0.05$. (A) Methemoglobin and Carboxyhemoglobin serum levels are increased by continuous administration of ISMN in a concentration-dependent manner. (B) Continuous administration of ISMN increased the serum levels of Methemoglobin and Carboxyhemoglobin ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid decreases their increased serum levels. (C) Continuous administration of ISMN decreased of oxygen saturation and oxygen content ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid restored the oxygen saturation and oxygen content concentrations. (D) Continuous administration of ISMN decreased the hemoglobin content ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of the L-ascorbic acid restored the decreased level of the hemoglobin content.

due to its very short half-life (Csonka et al., 2015). We chose the rat model since rats are more similar to human physiology that the mice model, making them better models for the studying pathological conditions in pre-clinical trials (Costa et al., 2019, Lu et al., 2019). Moreover, HSF diet induces hypertension rats; called spontaneously hypertensive rats (SHR) which are extensively used as an *in vivo* model for essential hypertension and cardiovascular disease (Plotnick et al., 2017). This SHR rat model has elevated basal myocardial NO content may be due to an increase in the protein-bound of dinitrosyl nonheme iron complexes which liberates the NO to the peripheral circulation that helps in treating the hypertensive state. Moreover, it has been previously published that SHR rats showed increased NO synthase activity III in the cardiac and aortic endothelium. Both of these two enzymes help in regulating vasoreactivity in the SHR rats (Plotnick et al., 2017).

Our data go along with the previously published data for using antioxidant for treating nitrate tolerance and they gave similar

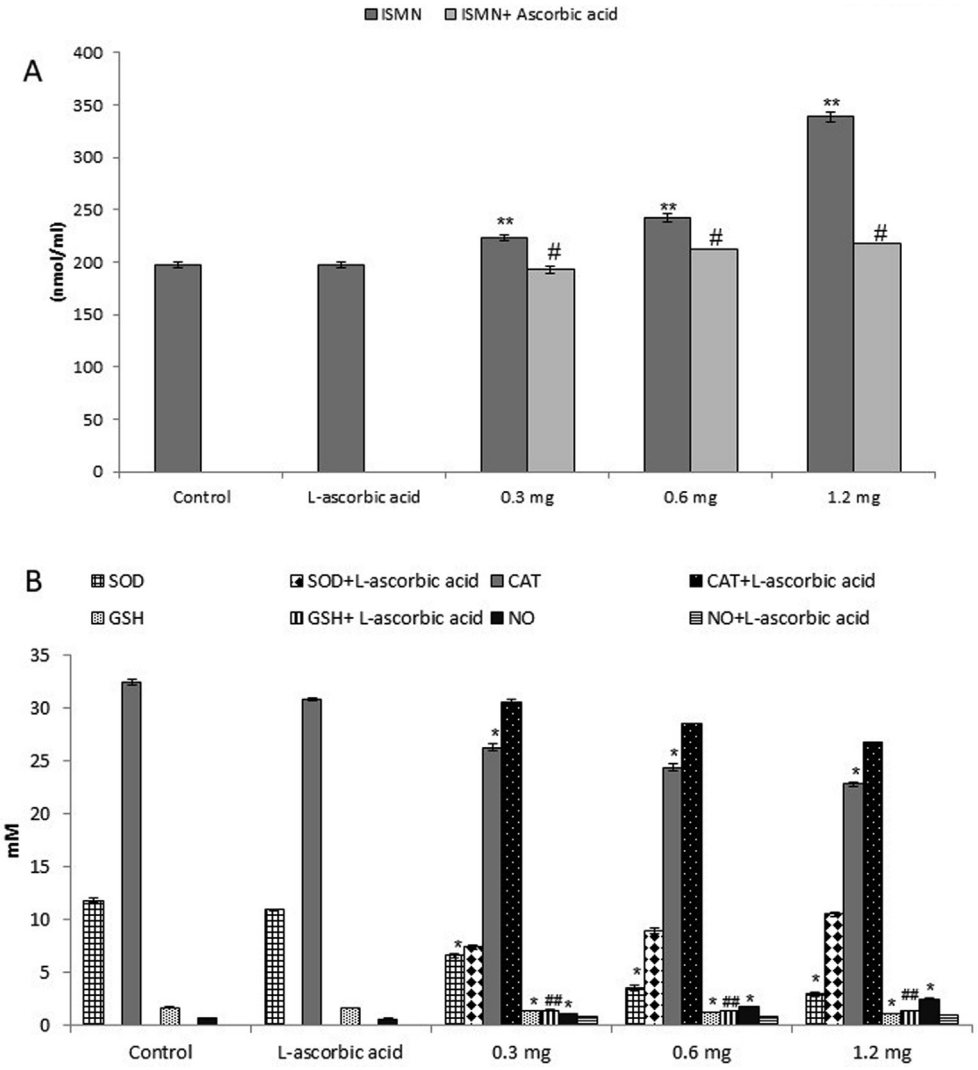

Fig. 3. Effect of continuous exposure to organic nitrates on the oxidative stress parameters. Data represented as means ± SD. n = 5–6. *: Significant decrease compared with control groups at $p < 0.05$. **: Significant increase compared with control groups at $p < 0.05$. #: Significant decrease compared with ISMN groups of the same dose at $p < 0.05$. ##: Significant increase compared with ISMN groups of the same dose at $p < 0.05$. MDA; Malondialdehyde, SOD; Superoxide dismutase, CAT; Catalase activity, GSH; Glutathione, NO; Nitrous Oxide. (A) Continuous exposure to ISMN increased Malondialdehyde (MDA) activity ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the increased level of MDA (B) Continuous exposure to ISMN leads decreased the activity of SOD, CAT, GSH and NO content level ($p < 0.05$) in a concentration-dependent manner. Concomitant administrated the decreased levels of SOD, CAT, GSH and NO content.

clinical data (Csonka et al., 2015, Stewart et al., 2018, Lu et al., 2019, Meera et al., 2020). Meanwhile, the authors stressed the oxidative stress markers. Here, we studied a very important stress marker; *nrf2*, has a great role in the antioxidant metabolism pathway and has an important role against reactive oxygen species producing the cellular injury in the myocardium muscles (Bryda, 2013). This gives a molecular explanation of the previously published results and quantitatively determined the dose of L-ascorbic acid that can be used clinically. Moreover, we studied the activity of the pro-inflammatory mediator gene; *NF-kB*. Our results showed another mechanism for the protective effect of L-ascorbic acid against NO deleterious effect on the myocardium tissue. Besides, our data on the apoptosis marker; *caspase-3*, gives an explanation for the end result of the protective effect of L-ascorbic acid on the myocardium tissue.

SOD, GSH, CAT and MDA are the first-line defense mechanisms against oxidative stress. They give an indication of the stressful conditions inside the cells (Lu et al., 2019). It has been previously reported a significant decrease in the activity of SOD, GSH and CAT, and a significant elevated level of plasma MDA (Plotnick et al., 2017). The suggested mechanism is that this may be an indirect effect of the significant increased NO plasma level or directly due to the significant elevated level of ROS and RNS. Meanwhile, the previous study (Plotnick et al., 2017) has not proved the tightly bound to that hypothesized subcellular event. In our study, we proved these suggestions by finding the dose-dependent response according to the plasma NO levels. Yet, L-ascorbic acid; a free radical scavenger, restored the change of these first-line defense mechanisms against oxidative stress. We measured the cellular levels of the pro-inflammatory cytokines marker (*NF-κB*), apoptosis markers (*caspase-3*) and mitochondrial oxidative stress marker (*nrf2*) in the myocardium muscles. *Nrf2* is a suppressive mitochondrial oxidative stress marker. If this marker is increased, this indicates an increased oxidative stress condition inside the cell. We found that ISMN elevated the cellular activity of *nrf2* in a concentration-dependent manner. Moreover, ISMN reduced the cellular activity of both the pro-inflammatory cytokines marker (*NF-κB*) and the apoptosis markers (*caspase-3*) in a dose-dependent manner. L-ascorbic acid succeeded to restore the cellular levels of the three markers in all tested concentra-

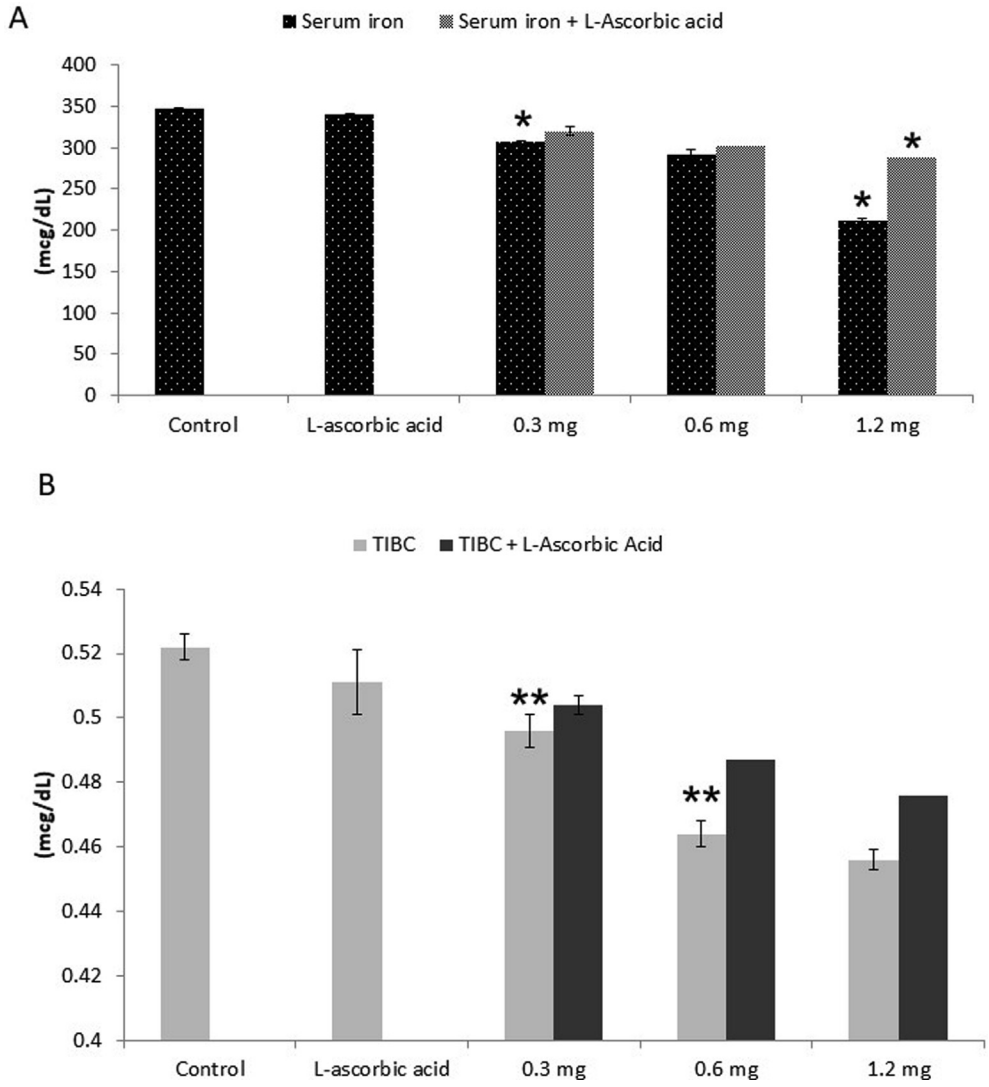

Fig. 4. Effect of continuous administration of organic nitrates on the iron content, total iron-binding capacity and inorganic phosphorus content. Data was presented as means ± SD. n = 4–6. *: Significant as compared with control group at $p < 0.05$. **: Significant as compared with control group at $p < 0.05$. (A) Continuous administration of organic nitrates reduced the serum iron level ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid restored the decreased level of the serum iron. (B) Continuous administration of organic nitrates leads to reduction of TIBC ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the decreased level of the TIBC.

tions of ISMN. We expected that the vasodilatory effect of ISMN will remain in chronic administration of ISMN due to the restoration of the endothelial function of the myocardium and no need to establish the NO free period. This nitrate free period for patients may cause an elevation of the risk of cardiovascular events during such nitrate-free periods, specifically in the early light phase.

Reduction of the plasma hemoglobin level and increase in the level of both methemoglobin and carboxyhemoglobin may be the consequence of the liberated nitric oxide and nitrate ions during isosorbide mononitrate metabolism which can oxidize hemoglobin to methemoglobin and carboxyhemoglobin (Stewart et al., 2018, Bryda, 2013). Increased methemoglobin levels can cause congenital enzymatic defects, variation in hemoglobin molecule (Amdahl et al., 2019, Kottaisamy et al., 2021). Methemoglobin does not bind to oxygen efficiently causing a reduction in the oxygen-carrying capacity of the blood and a reduction in saturated oxygen and oxygen content this decrease is significant in high doses of nitrates (Rochon et al., 2020). L-ascorbic acid protects the blood from the oxidant effect of nitric oxide (antioxidant effect) and induces decreasing the levels of met-hemoglobin concentration (Meera et al., 2020, Ahluwalia et al., 2021). This reduction in methemoglobin levels induced by L-ascorbic acid concluded that erythrocyte alone had a negligible ability to reduce methemoglobin in the absence of exogenous ascorbate. Ascorbic acid preserves the Hb in a reduced ferrous redox state (Rochon et al., 2020, Ahluwalia et al., 2021).

It has been previously reported that carboxyhemoglobin showed a significant increase with high doses of organic nitrates only (Leo et al., 2021). Our results showed that low doses of ISMN can significantly increase the plasma level of MetHb. This may be due to the impairment in the antioxidant enzymes defense system of the erythrocyte may cause an elevation of the abnormal hemoglobin derivatives as carboxyhemoglobin (Brunauer et al., 2016). An elevation in carboxyhemoglobin levels were noticed in all ISNN treated groups in a concentration-dependent manner. However, following L-ascorbic acid administration, a significant reduction of the carboxyhemoglobin was obtained in all treated groups. A

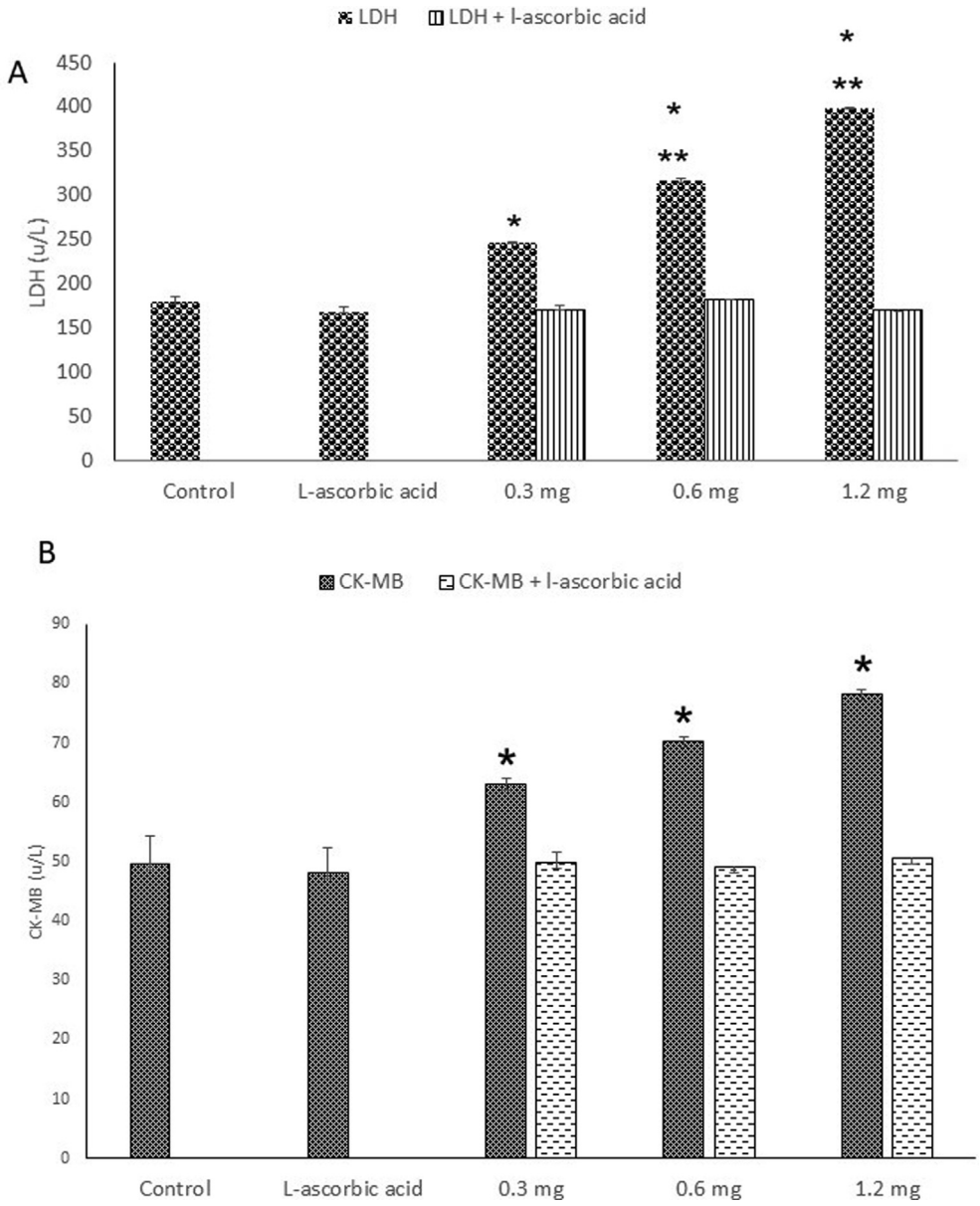

Fig. 5. Effect of continuous administration of organic nitrates on the cardiac biomarkers (lactate dehydrogenase and creatinine kinase-MB). Data was presented as means ± SD. n = 5–6. *: Significant as compared with control group at $p < 0.05$. **: Significant as compared with control group at $p < 0.05$. (A) Continuous administration of organic nitrates elevated the serum level of lactate dehydrogenase ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid restored the decreased level of the cardiac enzyme marker; lactate dehydrogenase (LDH). (B) Continuous administration of organic nitrates leads to an elevation of creatinine kinase – MB (CK-MB) ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the decreased level of the CK-MB.

reduction in hematocrit, RBC, and WBC may be a result of hemoglobin reduction and oxidative stress induced by nitric oxide (Brunauer et al., 2016, Tang et al., 2021).

The present study showed a significant decrease in both serum iron level and serum TIBC in all ISMN treated groups in a concentration-dependent manner. This result can be explained by the increased serum nitric oxide by the increased ISMN dose and by the induced oxidative stress decreasing the serum iron and TIBC (Luo et al., 2021). It has been reported that iron deficiency anemia increases through NO production, and elevated NO concentrations in iron deficiency anemia. This effect can be reversed by iron supplementation to regain its normal levels (Tang et al., 2021). In this study, an improvement in the serum iron level and TIBC was observed by the effect of L-ascorbic acid. This improvement may be due to the beneficial effect of L-ascorbic acid to enhance iron absorption (Tang et al., 2021, Luo et al., 2021). In our study, all groups treated with ISMN showed a significant increase in serum nitric oxide concentrations. It has been previously recorded that L-ascorbic acid significantly decreases nitric oxide concentrations.

L-ascorbic acid can decrease the accumulation of superoxide and peroxynitrite by directly scavenging superoxide (Leo et al., 2021). L-ascorbic acid safeguards against oxidative stress that induces pathological vasoconstriction and destruction of the endothelial barrier through blocking tetrahydrobiopterin oxidation, the cofactor of the endothelial nitric oxide synthase, thereby inhibiting endothelial nitric oxide depletion and endothelial nitric oxide synthase uncoupling. Ascorbate blocks inducible nitric oxide synthase preventing abundant production of NO (Morelli et al., 2020).

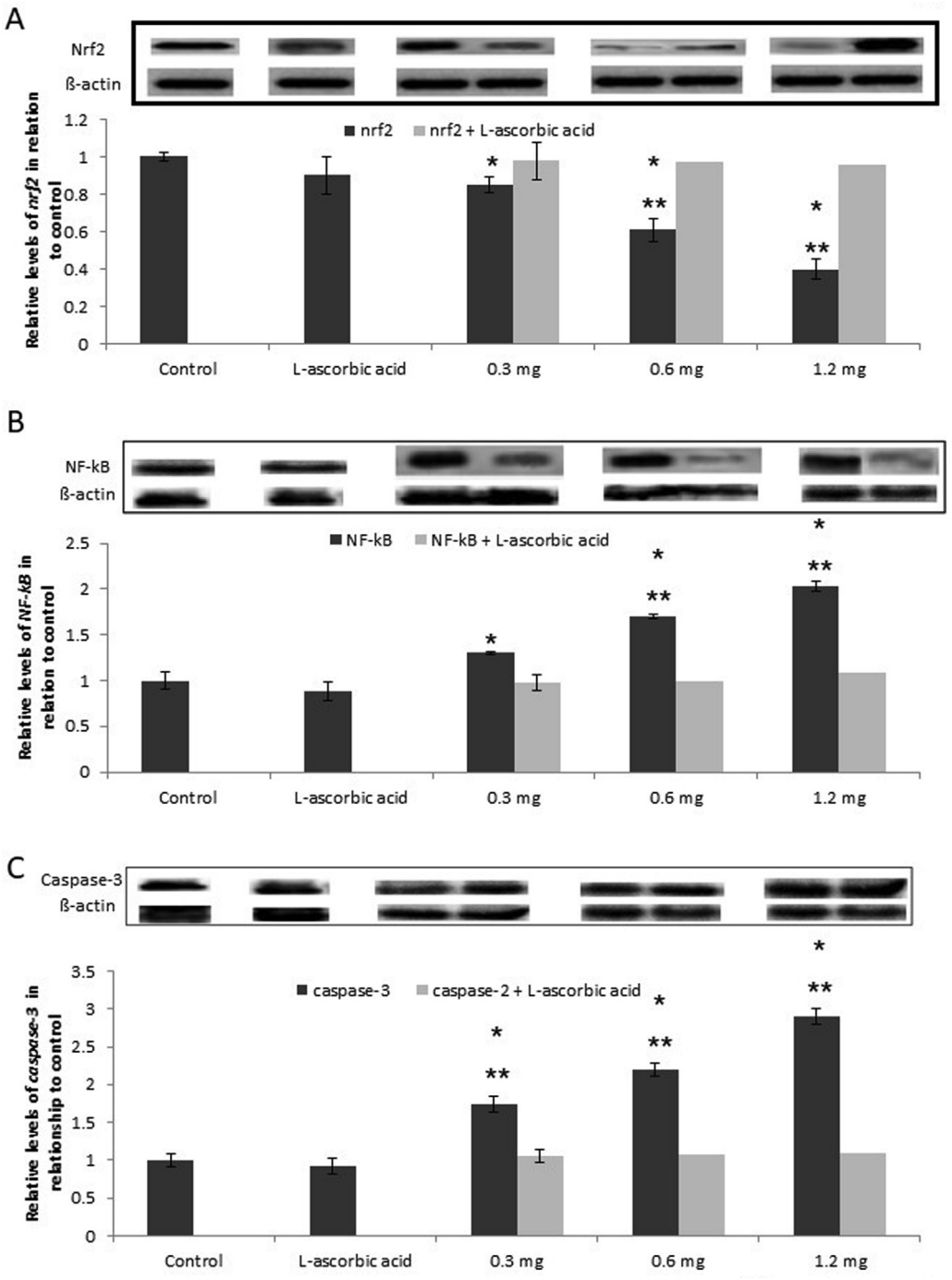

Fig. 6. Effect of continuous exposure to organic nitrates on the mitochondrial oxidative stress marker (*nrf2*), the pro-inflammatory cytokines marker (*NF-κB*) and apoptosis markers (*caspase-3*) concentrations in the myocardium muscles. Data was presented as means ± SD. n = 4–6. *: Significant as compared with control group at $p < 0.05$. **: Significant as compared with Ascorbic acid treated group at $p < 0.05$. (A) Continuous administration of organic nitrates reduced the relative activity level of *nrf2* ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid restored the decreased relative activity level of the *nrf2*. (B) Continuous administration of organic nitrates leads to an increase in the relative activity level of *NF-kB* ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the increased level of the *NF-kB*. (C) Continuous administration of organic nitrates leads to an increase in the relative activity level of *caspase-3* ($p < 0.05$) in a concentration-dependent manner. Concomitant administration of L-ascorbic acid leads to restoration the increased level of the *caspase-3*. Representative western blots of Nrf2, NF-kB and caspase-3 are shown for each group.

5. Conclusion

Our results explore the beneficial effects of L-ascorbic acid in nitrate therapy. Nitrates therapy for the treatment of cardiac problems induces elevated serum levels of lipid peroxidation, hemoglobin derivatives as methemoglobin and carboxyhemoglobin, rate of hemoglobin autoxidation, the cellular levels of the pro-inflammatory cytokines marker (*NF-κB*) and apoptosis markers (*caspase-3*) in the myocardium muscles in a dose-dependent manner. Meanwhile, Nitrates reduces the enzymatic effect of SOD, GSH and CAT accompanied by a decrease in the level of mitochondrial oxidative stress marker (*nrf2*) in the myocardium muscles and decreases both the serum iron and TIBC as well in a dose-dependent manner in the rat model.

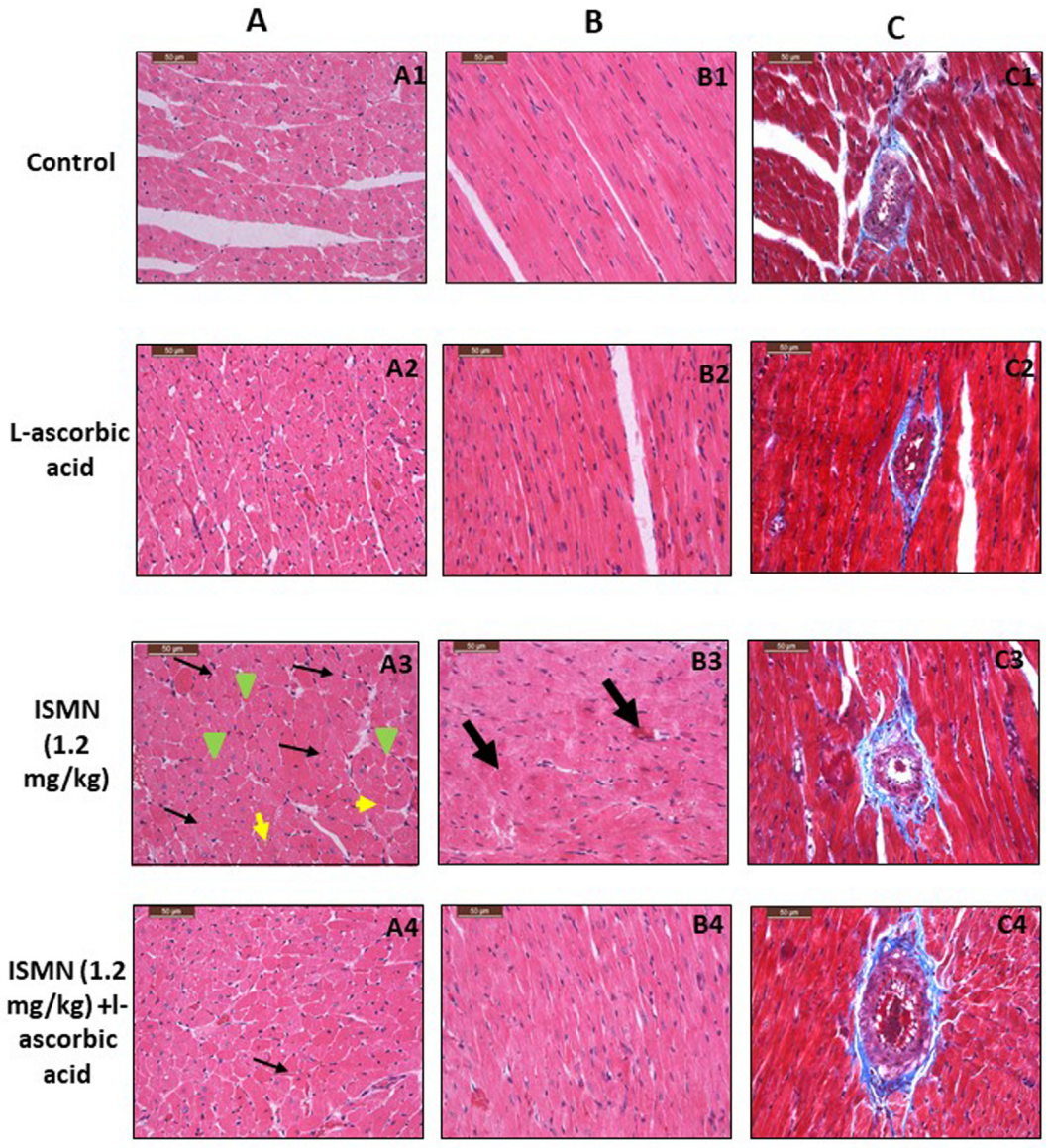

Fig. 7. Hematoxylin-eosin staining and Masson's trichrome staining of heart tissue (50X). Rat cardiac muscle tissue stained with H&E (A&B, columns) and Masson's trichrome (C, column). Normal structure of cardiac muscle in the control group and in the group administered L-ascorbic acid alone (A2&B2). The group administered ISMN (1.2 mg/kg body weight) (A3&B3) showed a moderate Zenker's necrosis (thin black arrows) accompanied by mononuclear inflammatory cells aggregation (green head arrows), cytoplasmic vacuoles (yellow arrows) (A3) and mild degeneration of myofibrils (thick black arrows) (B3). The group treated with ISMN (1.2 mg/kg) followed by L-ascorbic acid (A4&B4) showed mild recovery of myocardial lesions with normal myofibril structures (B4), but remained mild necrosis (black arrow), (A4). Masson's trichrome sections (C, colum) showed no fibrosis in the control group and in the group treated with L-ascorbic acid alone (C1&C2). The group treated with ISMN (1.2 mg/kg body weight) showed mild intermuscular fibrosis (C3). The groups co-administered ISMN (1.2 mg/kg body weight) with L-ascorbic acid showed a gradual decrease in intermuscular fibrosis (C4) (bars = 50).

Concomitant treatment with a moderate dose of L-ascorbic acid significantly ameliorates these changes for all examined parameters. A moderate dose (20 mg/kg body weight in the rat model) of L-ascorbic acid supplementation could emerge as an important therapeutic strategy to prevent organic nitrate oxidative stress and can reduce nitrate tolerance. It remains to try to include a moderate dose of L-ascorbic acid in nitrate therapy for patients to study the nitrate tolerance and the cardiotoxic biomarkers.

Disclosure

All authors declare no potential conflicts of interest, including any financial, personal or other relationships with other people or organizations within that could inappropriately influence, or be perceived to influence, this work.

Author contribution

Ahmed Mohsen Elsaid Hamdan; in vivo studies, paper drafting and revising the article critically for important intellectual content. Zuhair M. Mohammedsaleh; supervised and monitored all aspects of this study from conception of the idea to submission of the manuscript, Aalaa Aboelnour; contributed her statistical analysis and data interpretation, Sherif M.H. Elkhannishy; contributed byt the intellectual ability to conception the project design, biochemical studies and laboratory analysis. All authors contributed equally to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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