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Downregulation of *flt-1* and *HIF-1α* Gene Expression by Some Antioxidants in Rats Under Sodium Nitrite–Induced Hypoxic Stress

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Abstract

This study assessed the effect of L-arginine (L-argin), carnosine (carno), or their combination in the amelioration of certain biochemical indices induced in the liver of hypoxic rats. Hypoxia was induced via sodium nitrite (S.nit) injection at a dose of 75 mg/kg. Rats were administered L-argin (250 mg/kg) or carno (250 mg/kg), either alone or in combination, 24 hours and 1 hour prior to S.nit intoxication. Hypoxia significantly elevated serum alanine aminotransferase, in addition to a significant upregulation of hepatic heat shock protein 70 with concurrent reduction in the level of vascular endothelial growth factor. Moreover, hepatic vascular endothelial growth factor 1 (flt-1), hypoxia inducible factor-1α gene expression, and cytochrome P450 levels were elevated, compared with the normoxic group. The antioxidants, administered either alone or in combination, markedly downregulated all of the previously mentioned biomarkers, compared to the hypoxic rats. Histopathological examination revealed hepatocellular degeneration and nuclear pyknosis, in addition to inflammatory cellular infiltration in the hypoxic rats, whereas treatment with the *studied* antioxidants improved the liver architecture. The present data revealed the efficacy of L-argin and carno in ameliorating the hepatic damage induced via angiogenic markers in response to hypoxia, the combination regimen showing the superior effect.

Keywords

flt-1, Cyp-450, HSP-70, hypoxia

Introduction

Hypoxia is an important pathobiological process in many diseases that may cause changes in body functions, as it easily affects an organism's metabolism and impairs gas exchange between the organism and the environment. The oxygen concentration drops rapidly, whereas carbon dioxide concentration increases rapidly. Hypoxia might lead to functional impairment, disturbance of consciousness, reaction dullness, retardation of action, and damage to the learning-memory functions. Serious hypoxia might cause pathological damage or even death. Brain damage may be induced by energy exhaustion in brain cells, overexpression of excitatory amino acids, oxygen-free radical damage-induced apoptosis, and inflammation.[1]

Liver is a highly aerobic organ, whose viability and metabolism depend on oxygen availability. The oxygen consumption of the liver is 100 to 150 μmol O_2 per hour per gram of wet weight. For most circulation, blood flow is regulated primarily

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by oxygen demand. As the liver is an important site for first-pass clearance of hormones, stable hepatic blood flow prevents fluctuations in hormone levels. As sinusoidal blood moves through the hepatic lobules, the liver extracts oxygen, nutrients, bile acids, and hormones. In this way, sinusoidal blood flow and hepatic metabolism create gradients of oxygen, metabolites, and hormones between the periportal and pericentral regions of the liver lobule. Tissue responses to these gradients likely contribute to the development of biochemical differences between the hepatocytes in different regions of the hepatic lobule, such as the relative enrichment of gluconeogenesis, ureagenesis, and oxidative metabolism in periportal hepatocytes, and xenobiotic and glycolytic metabolism in pericentral hepatocytes.[2]

Sodium nitrite (S.nit) is an inorganic salt with either harmful or beneficial effect. It is named E250 in the food industry and utilized widely as a color fixative in fish, meat, and preservatives. Sodium nitrite is a vasodilator that aids in relieving pulmonary hypertension. Although S.nit is present in drinking water, diet is the main source of susceptibly for humans. Sodium nitrite reacts with hemoglobin (Hb), thus affecting hematopoiesis. Sodium nitrite toxicity causes methemoglobinemia, which decreases O_2 transport by Hb. The administration of S.nit causes oxidative stress, inflammation, and hypoxia.[3] In this context, S.nit represents an ideal chemical for a hypoxia study.

The aim of the present study was to estimate the effects of the antioxidants L-arginine (L-argin), carnosine (carno), and their combination on the inflammatory and angiogenic markers induced in the liver tissue of S.nit-intoxicated rats. L-arginine is a basic, natural amino acid that has important roles in many metabolic pathways. It is a precursor of proline, proteins, and glutamate.[4] L-arginine is essential for the biosynthesis of nitric oxide (NO), which plays a marked role in vasodilatation and regulation of the immune system.[5] Nitric oxide has anti-apoptotic actions.[6]

Carnosine is an important dipeptide of β-alanine and histidine that is markedly present in brain tissues and muscles; it can scavenge reactive oxygen species (ROS) and chelate divalent metal ions.[7] Moreover, it is recognized to have anti-inflammatory and antioxidant activities.[8]

Material and Methods

Chemicals

Carnosine, L-argin, S.nit, and all other chemicals used in this study were of a high analytical grade and products of Sigma-Aldrich Chemical Co (St Louis, Missouri). Sodium nitrite and carno were dissolved in normal saline, whereas L-argin was resuspended in 1% carboxymethyl cellulose in normal saline.

Animals and Treatments

Fifty male Wister albino rats, weighing 190 to 200 g, were obtained from the Experimental Animal Center, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The experimental animal protocol was approved by the Animal Care and Ethical Committee of the Faculty of Pharmacy, King Saud University. Animals were kept under standardized conditions (22°C ± 5°C, 55% ± 5% humidity, and 12 hours light/dark cycle). They were allowed free access to water and a chow diet.

After 1 week of acclimation, the rats were kept fasting overnight before treatment and divided into 5 groups (10 rats each) as follows:

Group 1: Served as a control group and received saline.
Group 2: Sodium nitrite–intoxicated rats received a single subcutaneous dose of 75 mg/kg.[9]
Group 3: Sodium nitrite–intoxicated rats were preinjected with L-argin at 200 mg/kg, intraperitoneally (IP).[7]
Group 4: Sodium nitrite–intoxicated rats were injected with carno at 250 mg/kg, IP.[10]
Group 5: Sodium nitrite–intoxicated rats were injected with a combination of L-argin at 250 mg/kg and carno at 250 mg/kg.

Of note, L-argin and carno were administered intraperitoneally, 24 hours and 1 hour prior to S.nit intoxication. One hour after S.nit injection, rats were killed under ether anesthesia, and then blood samples were collected and divided into 2 parts. One part was collected for Hb determination. The other part was allowed to coagulate and centrifuged for serum separation for use in serum biochemical analysis. After blood collection, the livers were removed and washed using a chilled saline solution, before homogenization in phosphate buffer to yield 20% homogenates. The homogenates were centrifuged for 20 minutes at 3000 rpm at 5°C, and the supernatants and the serum were stored at −80°C and used for further biochemical analysis. Three livers were placed overnight in 4% formalin for histopathological examination. Some of the liver tissues were kept under nitrogen for reverse transcription polymerase chain reactio analysis.

Biochemical Serum Analysis

Determination of Hb concentration. Hemoglobin concentration was determined colorimetrically using Drabkin reagent according to the method of Kjeldsberg.[11]

Determination of alanine aminotransferase. The alanine aminotransferase (ALT) level was determined using kits obtained from the Randox Company (Crumlin, County Antrim, UK), according to the method of Reitman and Frankle.[12]

Determination of vascular endothelial growth factor and cytochrome P450 levels. Vascular endothelial growth factor (VEGF) and cytochrome P450 (Cyp-450) were estimated using a highly sensitive, rat enzyme-linked immunosorbent assay (ELISA) kit (IBL International GmbH, Hamburg, Germany), following the instructions of the manufacturer.

Determination of heat shock protein 70 level. The heat shock protein 70 (HSP-70) level was estimated by an immunonephelometric assay (Dade Behring N Latex High Sensitivity CRPTM mono assay) on a Behring Nephelometer II analyzer. The level of VEGF was assayed by colorimetric, quantitative, sandwich ELISA (R&D Systems, United Kingdom) at 492 nm, following the manufacturer's instructions. For the quantitative determination of HSP-70, a sandwich rat HSP-70 ELISA Kit was used (Kamiya Biomedical, Washington, USA).

Real-time PCR for flt-1 and hypoxia inducible factor 1α determinations. Real-time PCR amplification and analysis were performed using an Applied Biosystems thermocycler with software version 3.1 (StepOne). The reaction contained SYBR Green Master Mix (Applied Biosystems). Gene-specific primer pairs are: hypoxia inducible factor-1α (HIF-1α): forward 5′-GTCGGACAGCCTCACCAAACAG-3′, reverse 5′-TAGGTAGTGAGCCACCAGTGTCC-3′ (198 base pairs); flt-1: forward 5′-CAAGGGACTCTACACTTGTC-3′,

reverse 5′-CCGAATAGCGAGCAGATTTC-3′ (267 base pairs) and were designed with Gene Runner Software (Hasting Software, Inc, Hasting, New York) from RNA sequences from the gene bank. All primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR was performed in a 25 μL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of each primer, and 2 μL of complementary DNA. Amplification conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of denaturation for 15 seconds, and annealing/extension at 60°C for 10 minutes. Data from real-time assays were calculated using the v1·7 sequence detection software from PE Biosystems (Foster City, California). Relative messenger RNA expression of the studied genes was calculated using the comparative *Ct* method. All values were normalized to β actin, which was used as the control housekeeping gene, and reported as fold change over background levels detected in the diseased groups.[13]

Histopathological Examination

Deparaffinized sections of 4 μm of liver tissues were stained with hematoxylin and eosin and examined under a light microscope.[14]

Statistical Analysis

Data are expressed as means ± standard error of the mean. Statistical analysis was performed using one-way analysis of variance by SPSS 12 program followed by post hoc test which was used to determine the differences between means of different groups. The level of significance was set at $P < .05$ using Tukey test.

Table 1. Blood Hemoglobin Concentration and Serum ALT Level in Control as well as Different Treated Groups.[a]

Groups	Hemoglobin (g/dL)	ALT (U/L)
Control	12.1 ± 0.72^b	58.94 ± 8.78^b
S.nit	5.5 ± 0.62^c	180.52 ± 5.24^c
S.nit and L-argin	10.2 ± 1.3^d	102.75 ± 10.31^d
S.nit and Carno	9.3 ± 0.22^d	115.91 ± 3.34^e
S.nit and L-argin and Carno	11.5 ± 0.9^b	95.44 ± 1.16^f

Abbreviations: ALT, alanine aminotransferase; carno, carnosine; L-argin, L-arginine; S.nit, sodium nitrite.
[a]Data are expressed as means ± standard error of the mean (n = 10). $P < .05$ is considered significant. Groups having the same letter (b, c, d, e, and f) are not significantly different, while those having different letters (b, c, d, e, and f) are significantly different.

Results

Biochemical Observations

A significant decrease in the Hb concentration was observed post S.nit toxicity treatment, compared with the control group (Table 1). Pretreatment of the hypoxic rats with L-argin and/or carno, either alone or in combination, markedly modulated the deviation in the blood Hb levels compared with the S.nit toxicity group. The serum ALT level increased owing to S.nit treatment. However, preadministration of L-argin and/or carno markedly reduced this increase, compared with the S.nit-intoxicated rats.

The hepatic levels of the Cyp450 and serum HSP-70 were significantly increased in the S.nit-intoxicated rats, compared with the control group (Figures 1 and 2). Preadministration of L-argin and/or carno markedly reduced this increase compared with the S.nit-intoxicated rats.

Real-time PCR indicated that S.nit administration induced a significant elevation in flt-1 and HIF-1α gene expression in liver tissues, compared with the control group, whereas L-argin, either alone or in combination with carno, downregulated flt-1 and HIF-1α expression, compared with the S.nit-intoxicated group ($P \leq .05$; Figures 1 and 2). Moreover, such treatment significantly reduced the Cyp-450 level compared with the S.nit-intoxicated animals (Figure 1). Administration of the combination of the aforementioned antioxidants to pre-S.nit-intoxicated rats decreased the VEGF, flt-1, HSP-70, HIF-1α, and Cyp-450 levels compared to S.nit-intoxicated group.

Histopathological Observation

The results of the biochemical markers were reinforced by histopathological examination of the liver tissues (Figure 3). Liver sections of the normal, control rats were characterized by the normal appearance of liver tissues, whereas rats exposed to S.nit toxicity exhibited liver degeneration and necrosis, in addition to binucleated cellular infiltration. The rats that received L-argin and/or carno showed a regression of the degeneration and cellular infiltration. Additional improvement of the

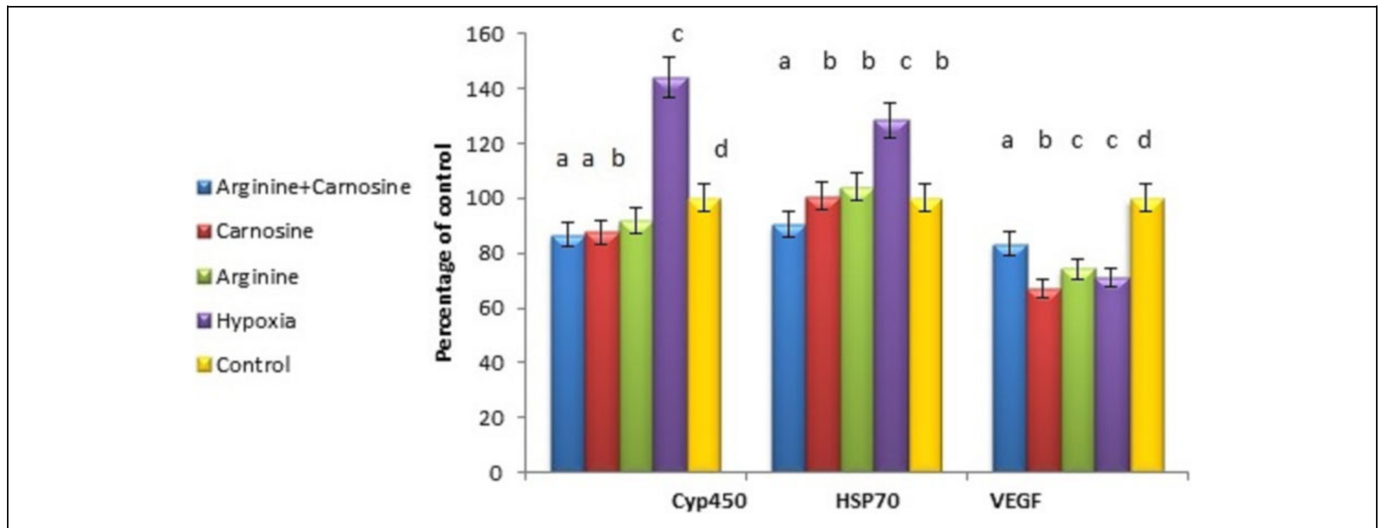

Figure 1. Effect of L-arginine, carnosine, and their combination on hepatic cytochrome P450 (Cyp-450), heat shock protein 70 (HSP 70), and vascular endothelial growth factor (VEGF) induced by hypoxia in rat's liver. Data are expressed as means ± standard error of the mean (SEM; n = 10). Value of $P < .05$ is considered significant. If the 2 bars take symbol "a," then they aren't significant, but if they take different letters, then they are significant.

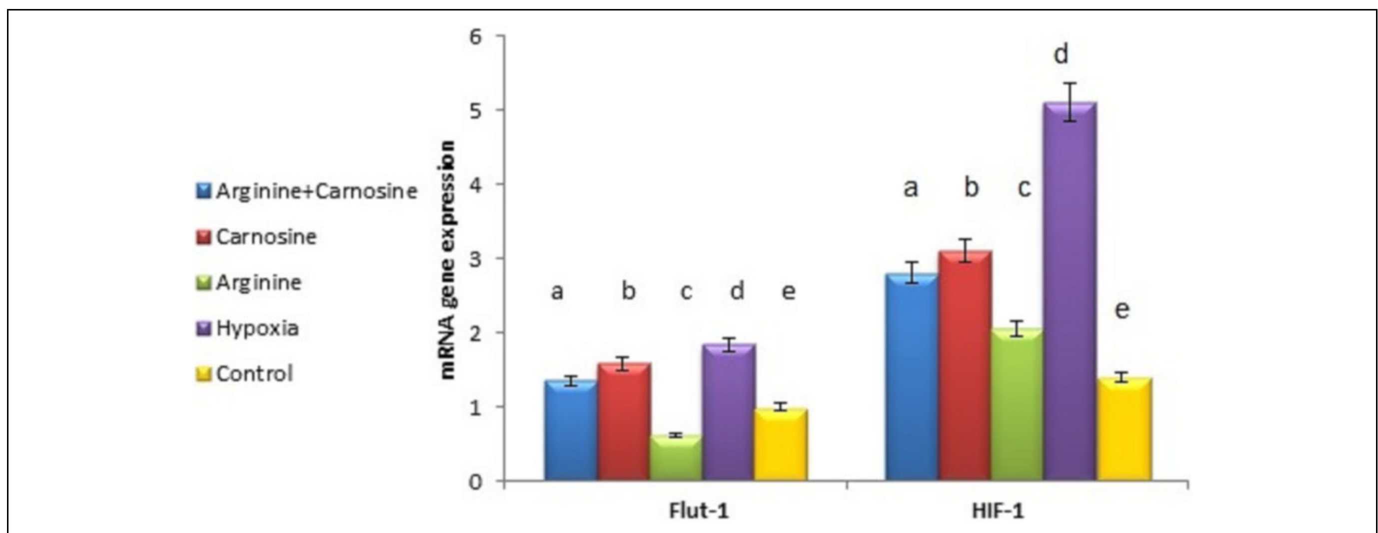

Figure 2. Effect of L-arginine, carnosine, and their combination on hepatic flt-1 and hypoxia inducible factor 1 (HIF-1) messenger RNA gene expression induced by hypoxia in rat's liver. Data are expressed as means ± standard error of the mean (SEM; n = 10). P value <.05 is considered significant. Groups having the same letter are not significantly different, while those having different letters are significantly different. If the 2 bars take symbol "a," then they aren't significant, but if they take different letters, then they are significant.

pathological changes of the liver architecture was obvious in the rats that received the combination of L-argin and Carno.

Discussion

Sodium nitrite is a food additive and also used as disinfectant in drinking water.[15] It induces lung, liver, heart, and brain injuries through the interaction of NO_2 with amines or amides present in the diet to induce toxic nitrosamines and nitrosamides, respectively.[16] Sodium nitrite induced in hepatic injury is related to oxidative stress, inflammation, and methemoglobinemia.[17]

Herein, the protective role of L-argin or carno and their combination against liver injury induced by S.nit was examined in rats. Sodium nitrite significantly decreased blood Hb level compared with the control group. The decrease in the Hb concentration may be due to elevated peroxide concentrations during inflammation. Sodium nitrite reacts with Hb, leading to methemoglobinemia which reduces O_2 transport causing organ's injury.[18]

An imbalance of the redox state induced by S.nit is considered the main cause of the alteration in liver function.[2] Pretreatment with L-argin, carno, or their combination markedly mitigated the reduced blood Hb matched with S.nit-intoxicated rats, which was attributed to their marked antioxidant index.[19]

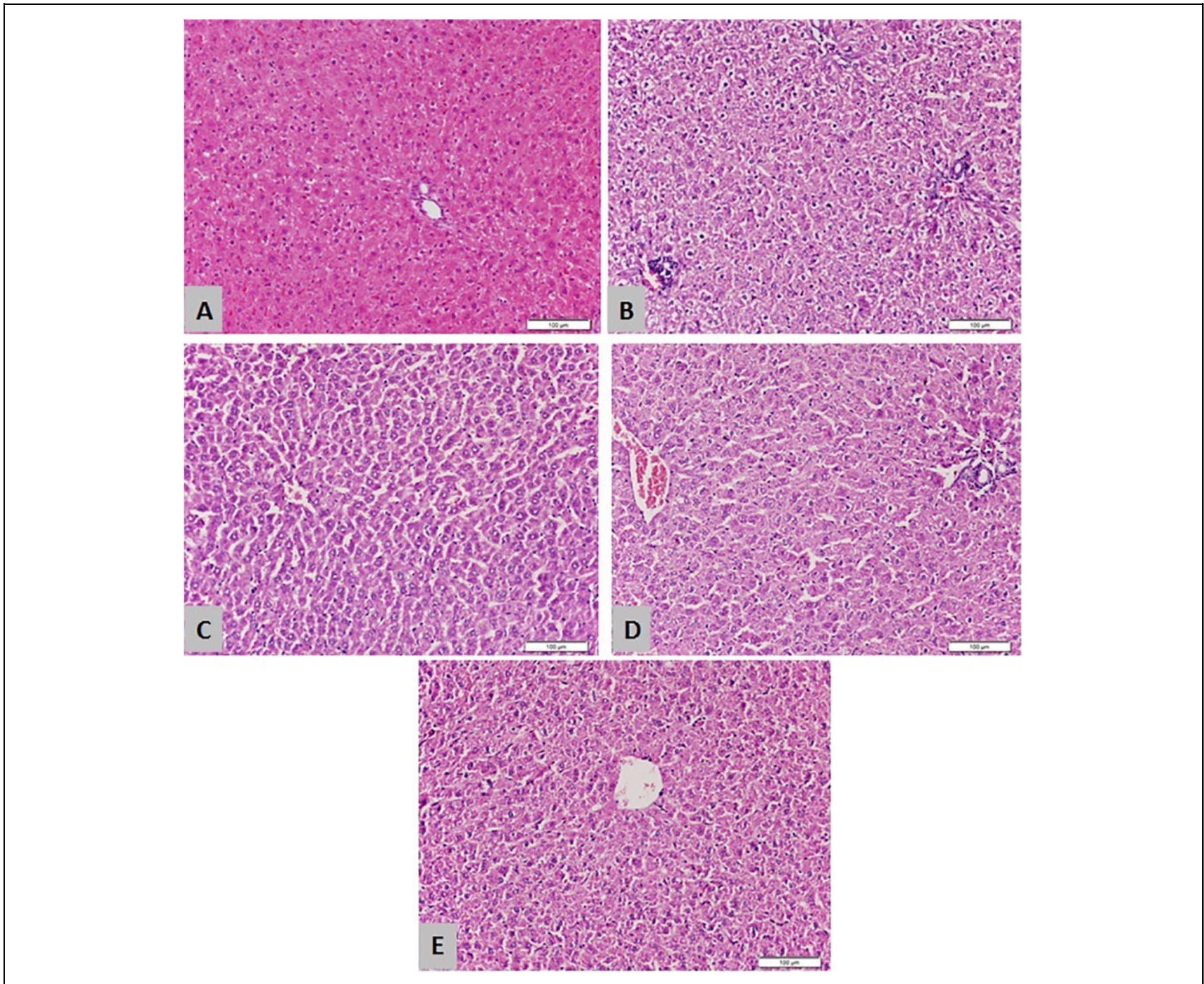

Figure 3. Light photomicrographs from liver of rat stained with hematoxylin and eosin (scale bar: 100 μm), in which (A) represent normal hepatocytes and portal area. B, Section of liver from rat exposed to hypoxia showing marked hepatocellular degeneration both cytoplasmic and nuclear in a form of pyknosis. Also, there are areas of cellular infiltration. (C), (D), and (E) are sections of liver from rat exposed to hypoxia and received L-arginine, carnosine, and combination of both, respectively, showing marked improvement in the cellular degeneration and a marked decrease in the cellular infiltration

In the current study, administration of S.nit caused marked increase in the hepatic marker (ALT), inflammatory markers (HSP-70), HIF-1α, Cyp-450, and Flt-1. These results are in harmony with those of Al-Gayyar, which revealed that S.nit exhibited oxidative stress, inflammation, as well as hypoxic stress which may lead to pulmonary and hepatic damage.[3]

Heat shock proteins are the first defense mechanism against cellular injury to restore homeostasis.[20] Herein, pretreatment with L-argin or carno and their combination markedly reduced the elevated ALT, HSP-70, HIF-1α, Cyp-450, and Flt-1. In harmony, it was reported that L-argin is essential for the synthesis of NO, which is a neurotransmitter, antiapoptotic, and a vasodilator.[5] Moreover, carno chelates divalent metal ions and scavenges ROS[7] and it has antioxidant and anti-inflammatory actions.[8]

It was reported that HIF-1α is activated during hypoxic state via induction of flt-1 and Inducible nitric oxide synthase (iNOS) that induce angiogenesis. Herein, S.nit-intoxication caused a remarkable upregulation of VEGF and HIF-1α levels, whereas administration of carno or L-argin and their combination downregulated the aforementioned measured parameters. It was previously reported that an increase in pro-inflammatory mediators activated VEGF expression which supported angiogenesis.[21] Ali et al reported that carno and L-argin were able to reduce VEGF expression.[22]

It was documented that HSPs are expressed by genes that control HIF-1α pathway in hypoxic situation, and it act on proteins misfolded by oxidative stress.[23] In the present study, HSP-70 level was significantly higher in the S.nit-intoxicated

group matched with normoxic one; this finding was in agreement with the results of Tsuchida et al,[24] which suggested that under hypoxic condition HIF-1α induced HSP-70 overexpression in chondrocytes.

The obtained results demonstrated that pretreatment with L-argin or carno and their combination downregulated the marked elevation in HSP-70, compared to the S.nit-intoxicated animals. The combination regimen was the most effective in restoring the levels of the previously mentioned parameters; the same results was proved by Wu et al.[25]

It was found that *protein kinase B* (AKT) is involved in angiogenesis regulation.[26] Hypoxia inducible factor 1α expression was induced due to the decreased oxygen tension and was modulated through AKT signaling pathway.[27]

The results of the present work revealed that S.nit induced a significant elevation in hepatic flt-1 gene expression and Cyp-450 level. However, administration of L-argin, carno, or their combination significantly reduced the levels of these altered parameters.

Cytochrome proteins are responsible for the metabolism of endogenous steroids, as well as exogenous compounds, including drugs and xenobiotic. This metabolism can lead to metabolic activation of toxic or carcinogenic metabolites. Generally, inhibition of Cyp isoforms can decrease metabolism and hepatic clearance of the substrates that are metabolized by that specific isoform.

Herein, it was found that pretreatment with L-argin, carno, or their combination may protect liver cells from S.nit-induced toxicity; the combination of L-argin and carno decreased Cyp-450 expression, through the inhibition of inflammatory and apoptotic pathways. Our results are in parallel with the results of Jie et al,[28] who reported that carno can downregulate Cyp-450 expression, and also in agreement with Shen and Hua,[29] who reported that L-argin suppressed Cyp-450 level.

Vascular endothelial growth factor receptor (VEGFR) has been shown to play major roles not only physiologically but also in pathological angiogenesis, such as cancer. Vascular endothelial growth factor A regulates angiogenesis and vascular permeability by activating 2 receptors: VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice). On the other hand, VEGF-C/VEGF-D and their receptor, VEGFR-3 (Flt-4), mainly regulate lymphangiogenesis.[30]

The flt-1 and Smad signaling pathways are crucial for myofibroblast activation.[31] The obtained results showed that S.nit toxicity produced a marked upregulation in the Flt-1 level, which was downregulated by L-argin, carno, or their combination. Abdelazim et al[32] reported that carno downregulated the overexpression of flt-1, Smad-2, IL-6, and TNF-α and the angiogenic factor VEGF in response to n-TiO_2 intoxication.

It was clear that liver sections of the hypoxic rats were characterized by hepatocellular degeneration, nuclear pyknosis, and inflammatory cellular infiltration. Rats received L-argin and/or carno showed a regression of the cellular degeneration and cellular infiltration. A marked improvement of the pathological changes was obvious in the rats received the combination of L-argin and carno. The present study revealed that L-argin and carno significantly reduced the previously mentioned angiogenic and inflammatory markers, the combination regimen demonstrating a superior effect.

Conclusion

The present study revealed that L-argin and carno significantly reduced the previously mentioned angiogenic and inflammatory markers, the combination regimen demonstrating a superior effect.

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Declaration of Conflicting Interests

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