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Original Article

Effects of L-arginine on the release of heme oxygenase-1 on ischemia-reperfusion induced acute pancreatitis in rats

Kursat Dikmen[1], Hasan Bostanci[1], Huseyin Gobut[2], Aydin Yavuz[2], Ozlem Gulbahar[3], Nalan Akyurek[4], Osman Yuksel[1]

[1]Department of General Surgery, Medical Faculty, Gazi University, Ankara, Turkey; [2]Department of General Surgery, Yeni Mahalle Training and Education Hospital, Yildirim Beyazıt University, Ankara, Turkey; [3]Department of Biochemistry, Medical Faculty, Gazi University, Ankara, Turkey; [4]Department of Pathology, Medical Faculty, Gazi University, Ankara, Turkey

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Abstract: Background: Ischemia-reperfusion (I/R) is a causative factor in the pathogenesis of acute pancreatitis. L-arginine plays a key role in the relationship between microcirculatory disorders and I/R injuries. Heme oxygenase-1 (HO-1) has been identified as a stress protein induced in many cell types by various stimulants, such as oxidative stress. Aim: The present study aimed to investigate the effects of L-arginine on HO-1 in pancreatitis resulting from ischemia and reperfusion. Materials and Methods: Pancreatic arterial vessels were prepared and clamped for 1 hour, then released after 3 hours. Animals were divided into 5 groups, sham, L-arginine+ischemia without reperfusion, saline+ischemia without reperfusion, L-arginine+ischemia with reperfusion, and saline+ischemia with reperfusion. Blood was collected for amylase and myeloperoxidase (MPO) and pancreatic tissue was collected for superoxide dismutase (SOD), malondialdehyde (MDA), heme-oxygenase-1 (HO-1), and histopathologic grading of pancreatic injuries. Results: Levels of amylase, MPO, SOD, and MDA in the L-arginine+ischemia without reperfusion and L-arginine+I/R groups were lower than in saline+ischemia without reperfusion and saline+I/R groups ($p<0.05$). In the L-arginine+I/R group, these parameters were lower than the L-arginine+ischemia without reperfusion group ($p<0.05$). In the saline+ischemia without reperfusion group, MPO, SOD, and MDA levels were significantly higher, compared to the saline+I/R group ($p<0.05$). HO-1 expression was significantly higher in the L-arginine treated groups. It was highest in the L-arginine+I/R group ($p<0.05$). Histopathological findings also supported the protective roles of L-arginine. Conclusion: Present data suggests that L-arginine, inducing HO-1 expression, could be useful in preventing oxidative damage associated with I/R induced pancreatitis.

Keywords: Ischemia/reperfusion induced pancreatitis, L-arginine, heme-oxygenase

Introduction

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas, usually affecting many organ systems. It has been reported that high mortality rates have decreased in recent years for this disease, but mortality rates are still 20-40% in cases of severe pancreatitis [1-3]. This is due to the complexity of the etiology and the inadequacy of targeted therapies due to an incomplete understanding of pathophysiology. Pathophysiological processes, such as inflammation, apoptosis, necrosis, and oxidative stress, have been associated with AP and are responsible for structural and morphological changes in the pancreatic tissue [4]. Ischemia is another important cause of the underlying mechanisms of AP pathophysiology, often causing the activation of pancreatic enzymes. The pancreas is a particularly sensitive organ for hypotension. It has been reported that histological tissue damage exists in cases of transient ischemia of about 40 minutes [5]. In particular, incidence of pancreatitis due to hypoperfusion of the splenic region during cardiac surgery is approximately 16%. This is more evident after sepsis, septic shock, and pancreas transplantation [6, 7]. These findings support the hypothesis that the mechanisms responsible for primary reperfusion injuries after isch-

emia include microcirculatory disorders. Thus, besides microcirculatory disorders, vasoconstriction, intravascular coagulation, the formation of free radicals, and release of pro-inflammatory mediators, intensify tissue damage.

Heme oxygenase-1 (HO-1) plays an active role in the process of protecting cells in almost all types of cellular stress [8]. HO-1 has been shown to be important in the protection of cells, tissues, and even organs due to cytoprotective, anti-inflammatory, anti-proliferative, and anti-apoptotic effects of HO-1 in clinical and experimental studies with various HO-1 related diseases [9, 10]. In these studies, it was demonstrated that HO-1 was upregulated rapidly and highly. Moreover, it has been reported that HO-1 metabolites reduced mortality in experimental pancreatitis models [11].

L-arginine (Arg) is a semi-essential amino acid found in protein structures of all living things, a precursor for the production of nitric oxide (NO). NO has a variety of biological functions on microcirculation in a multi-organism. Vasodilatation has protective effects on various organs, such as the pancreas and liver, by regulating local blood flow, inhibiting platelet aggregation and leukocyte adhesion, clearing free oxygen radicals, decreasing blood viscosity, and correcting microcirculatory impairment [12-15]. There are many different studies reporting that pancreatic NO levels decrease or increase during acute pancreatitis [7, 15, 16]. However, in an experimental acute pancreatitis model, it has been reported that, in rats given L-arginine, the severity of pancreatitis regressed and pancreatic blood flow improved due to the pruritus [17]. L-arginine may be involved in the release of HO-1 by regulating nitric oxide-associated pathways [18]. The present study aimed to investigate the efficacy of L-arginine on HO-1 in a pancreatitis model that was formed by I/R injury, showing changes in pancreatitis levels.

Materials and methods

Animals and surgical procedure

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Gazi University Rectorship Experiment Animals Ethics Committee. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23°C, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 1 week prior to experimentation. In this study, 50 female Wistar-albino rats, weighing 300-350 g, were used. All experiments were performed with rats that had fasted for 12 hours before surgery. Anesthesia was achieved by injecting Xylazine hydrochloride (Rompun, Bayer HealthCare) at a dose of 5 mg/kg, in addition to an intramuscular ketamine hydrochloride (Ketalar, Parke Davis and Eczacibasi, Istanbul) dose of 50 mg/kg. After anesthesia, abdomens of the rats were opened with a longitudinal incision. Preparation of the pancreas was performed, as described in previous studies [19]. After the preparation of the pancreas, the left gastric artery, gastroduodenal artery, splenic artery, and pancreaticoduodenal arteries feeding the pancreas with microvascular clamps were clipped and pancreatic tissue ischemia was performed for 1 hour. Following 1 hour of tissue ischemia, clips were removed and reperfusion was allowed for 3 hours. Blood and tissue samples were taken after ischemia and I/R.

Experimental design

Rats were randomly divided into 5 groups, with 10 rats in each group. Rats in Group I were subjected to laparotomy only without manipulation of the pancreas. Rats in Group II (ischemia+L-arginine) were subjected to pancreatic ischemia procedure plus administration of 400 mg/kg L-arginine via intraperitoneal. Rats in Group III (ischemia+Saline solution) were subjected to pancreatic ischemia procedure plus intraperitoneal administration of saline solution. Rats in Group IV (ischemia/reperfusion+L-arginine) were subjected to pancreatic I/R procedure plus administration of 400 mg/kg L-arginine via intraperitoneal. Rats in Group V (I/R+Saline solution) were subjected to pancreatic I/R procedure plus intraperitoneal administration of saline solution. Rats in Groups II and III were subjected to laparotomies and 1 hour of ischemia. Rats in Groups IV and V were subjected to laparotomies and 1 hour of ischemia, followed by 3 hours of reperfusion.

Analysis of biochemical indexes in serum and pancreas

An automated biochemistry analyzer examined amylase activity. HO-1 in serum was assessed using ELISA kits. Malandialdehyde (MDA), Su-

Table 1. Results of biological analysis of all groups (Mean ± SD)

	Sham	Arginine+Ischemia	Saline+Ischemia	Arginine+I/R	Saline+I/R
Amylase (mU/L)	151±23	760±76*	800±169*	623±107*,#,¥	842±118*,#
MPO (ng/mL)	114±23	267±37*	282±87*	165± 58*,#,¥	306±34*,#
SOD (U/mg)	11±3.7	29±6.1*	26±5.8*	31±5.9*,#,¥	21±4.7*,#
MDA (nmol/mg)	1.1±0.3	2.92±0.95*	3.81±0.82*	2.41±0.83*,#,¥	4.11±0.79*,#
HO-1 (pmol/mg/saat)	41±6	153±16	121±13	212±21#,¥	98±10#

MPO: Myeloperoxidase, SOD: Superoxide dismutase, MDA: Malandialdehyde, HO: Heme-oxygenase. *P<0.05; when compared to sham group. #P<0.05; when the reperfusion groups were compared with the non-reperfusion groups. ¥P<0.05; When the reperfusion and arginine groups were compared with the groups that were re-perfused but not given arginine.

peroxide dismutase (SPO), and Myeloperoxidase (MPO) levels in the pancreas were also evaluated using specific kits, according to manufacturer instructions.

Histological analysis

Routine formalin-fixed and paraffin-embedded (FFPE) pancreatic tissue sections (paraffin sections) were examined histologically using routine diagnostic laboratory methods. The severity of pancreatitis was determined according to Schmidt [20], who described a scoring system. This scoring system includes the graded assessment of pancreatic edema, inflammatory infiltration, pancreatic necrosis, and pancreatic aciner cell degeneration. A scale of 0-4 was used.

Statistical analysis

Evaluated parameters were computerized and evaluated using IBM SPSS ver. 20.0 (IBM Co., Armonk, NY, USA). Results are reported as the mean and standard deviation (SD). Statistical data should be expressed as mean ± SD or mean ± SE. Continuous variables were analyzed using Mann-Whitney's U-Test and histological analysis was determined using the Kruskal-Wallis test. *P*-values <0.05 indicate statistical significance.

Results

Serum levels of amylase for all groups are shown on **Table 1**. There was a significant increase in serum amylase levels in pancreatic ischemia groups (Group 2 and 3) and pancreatic ischemia/reperfusion groups (Group 4 and 5), compared to the sham group. There was no statistically significant differences in serum amylase levels between groups 2 and 3. A significant reduction in serum amylase levels was observed in the Arg+I/R group, compared with the Arg+Ischemia group. A significant reduction in serum amylase levels was observed in the Arg+I/R group, compared with the saline+I/R group (**Figure 1A**).

Levels of MPO and MDA for all study groups are shown in **Table 1**. MPO and MDA levels were significantly lower in the sham-operated group, compared with other groups. Levels were lower in the pancreatic ischemia+Arg treated group than the pancreatic ischemia+saline treated group, but these differences were not statistically significant. However, MPO and MDA levels were obviously lower in the I/R+Arg treated group than in the pancreatic ischemia+Arg group and pancreatic saline+I/R group, but these differences were not statistically significant (**Figure 1B** and **1C**).

Levels of SOD enzyme for all study groups are shown in **Table 1**. SOD levels were significantly higher in the sham-operated group, compared with other groups. In terms of SOD enzyme activity, SOD enzyme levels were found higher in the ischemia+saline group than the I/R+saline group and ischemia+saline group. This increase was found to be statistically significant. Similarly, when the I/R+Arg group was compared with the ischemia+Arg group in terms of SOD enzyme activities, the highest values were found in the I/R+Arg group. This height was statistically significant (**Figure 1D**).

Present data showed a significant increase of HO-1 expression in only ischemia rats and pancreatic I/R rats, compared with the sham-operated group. There was a significance increase in HO-1 levels in pancreatic I/R groups treated with saline or L-arginine, compared to pancreatic ischemia groups treated with saline or L-arginine. Treatment with L-arginine significantly

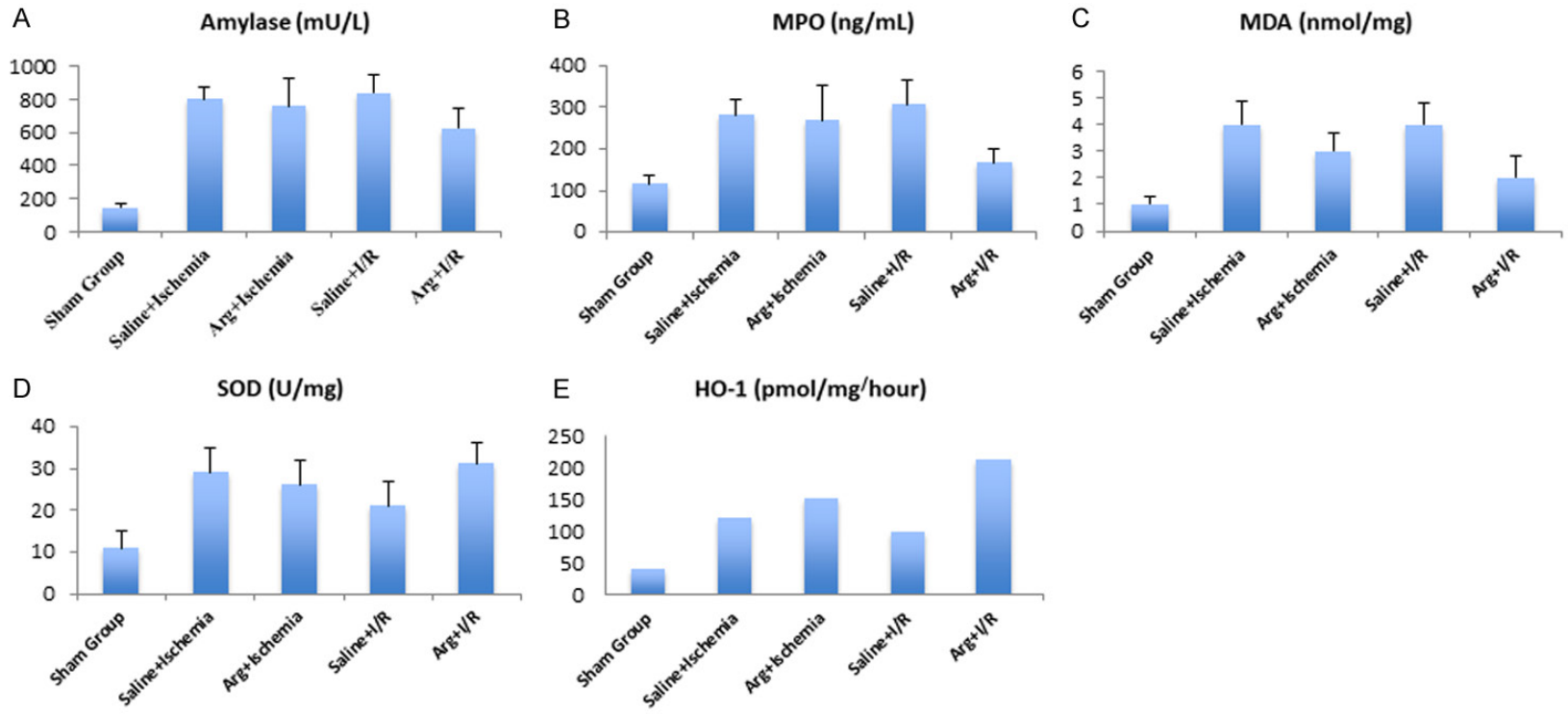

Figure 1. L-arginin ameliorates the severity of acute pancreatitis. Pancreatitis was induced in rats by ishemia/reperfusion in rats. (A) Serum amylase, (B) Myeloperoxidase (MPO) activity, (C) Malandialdehyde (MDA) levels, (D) Superoxide dismutase (SOD) enzyme activity and (E) Pancreatic Heme-oxygenase (HO)-1 activity.

Table 2. Results of histopathological analysis showing pancreatitis severity (Mean ± SD)

	Sham	Arginine+Ischemia	Saline+Ischemia	Arginine+I/R	Saline+I/R
Edema	0.65±0.55	1.87±1.18	2.37±0.69	1.13±0.44*	2.66±0.86
Aciner cell degeneration	0	0.6±0.88	0.9±0.92	0.4±0.7*	1.12±1.32
Necrosis	0	0.81±1.19	0.87±1.03	0.31±0.70*	0.93±1.14
Inflammation	0.06±0.17	0.87±0.5	0.93±0.97	0.62±1.27*	2.25±1.51

*P<0.05; When compared L-arginin+I/R and Saline I/R group.

increased expression of HO-1 in the pancreatic I/R group, compared to the ischemia group (**Figure 1E**).

Animals from the sham-operated group presented low pathological changes. Histopathological scores of the groups are summarized in **Table 2**. Pancreatic specimens from the saline+I/R group showed the strongest histological alterations, such as pancreatic asiner cell degeneration, necrosis, edema, and inflammation. In contrast, histopathological evidence of pancreatic damage was reduced in the ischemia+Arg group, compared with the ischemia+Arg group, but these differences were not statistically significance. The degree of pancreatic cell degeneration, pancreatic edema, necrosis, and inflammation showed significant differences between groups treated with L-arginine with or without reperfusion. Also, there were no significant differences in histopathological alterations between groups treated with saline with or without ischemia groups.

Discussion

The present study investigated the damaging effects of acute pancreatitis L-arginine induced by experimental ischemia/reperfusion injury via HO-1 enzymes. In experimental studies, it has been reported that pancreatic ischemic ischemia may cause primer in the development of acute pancreatitis [21]. Reperfusion of ischemic tissue causes endothelium-dependent dilatation of arterioles, capillaries leading to the formation of leukocyte plugs, increased fluid infiltration, escape of vascular proteins out of the vein, and ultimately impaired micro-vascular function. Thus, a number of oxidative stress mechanisms trigger tissue damage, such as vasoconstriction, formation of free oxygen radicals, intravascular coagulation, and release of pro-inflammatory cytokines [22].

Oxidative stress associated with I/R has important roles in the pathogenesis of pancreatitis development [23]. Oxidative stress is defined as the antioxidant deterioration of the balance between pro-oxidants products and antioxidants. Increased oxidative stress causes significant changes in cellular enzyme activities, membrane structures, membrane transport, antioxidant defense systems, and synthesis of heat shock proteins, in which HO-1 is involved. It has been shown that HO-1, an inducible form of the HO enzyme system, has protective effects in the *in vitro* and *in vivo* cellular damage processes [19]. This enzyme system has effects, such as anti-inflammatory, anti-oxidative, anti-proliferative, anti-thrombotic, and vasodilator with vital prescription for metabolism [24]. In addition, this enzyme reduces leukocyte activation and inhibits leukocyte adhesion to endothelial cells [19]. Adhesion of leukocytes to the endothelial cells or activation of leukocytes is extremely important in the development of micro-vascular insufficiency, one of the main causes of the pancreatitis pattern. HO-1 is induced by environmental factors, such as radiation and oxidative stress, and by various molecules, such as cytokines and inflammatory mediators [25, 26]. Previous experimental studies have shown that lipopolysaccharide increases HO-1 gene expression [27, 28]. Indeed, in some studies, more hepatocellular necrosis has been shown to cause the release of pro-inflammatory cytokines and more mortality in HO-1 deficient mice, compared to lipopolysaccharide wild-type mice [29]. There are also studies showing that HO-1 is induced at high rate by agents causing oxidative stress [30]. In this study, it was found that HO-1 concentrations in saline+I/R damaged rats were lower than in saline+ischemic rats. When tissue damage was thought to increase more with I/R, the protective effects were expected to be the highest activity of the known HO-1 enzyme activity in the saline+I/R group. However, present results were the opposite of this expectation. Inhibition of HO-1 enzyme activity or lack

of a stimulus to activate this system may be the cause of this condition. In the present study, L-arginine administration significantly increased HO-1 activity.

Nitric oxide is synthesized from L-arginine via nitric oxide synthase enzyme in vascular endothelial cells. Nitric oxide plays an active role in anti-arteriosclerotic, anti-oxidant, and cytoprotective effects, as well as in various physiological and pathological processes, which are extremely important in the regulation of blood vessels [31]. It also regulates processes, such as inhibiting leukocyte adhesion and platelet aggregation and reducing inflammatory cytokine response [32]. However, NO, a highly reactive molecule, is at very low levels under normal conditions and has a bi-directional effect due to polysaccharides. In other words, low concentrations of NO have been shown to be cytoprotective against oxidative stress-induced cell death, whereas high concentrations are cytotoxic to many types of cells [31]. There are studies with different conclusions reporting that levels of pancreatic NO are diminishing or increasing [7]. In an experimental study, it has been reported that pancreatitis was regressed and pancreatic blood flow was improved by administration of L-arginine to pancreatitis-treated rats [7]. In another study by Atsushi and colleagues, it was shown that nitric oxide-containing NOC12 administration in a low amount induced HO-1 enzymes at a significant level [33]. In addition to this work, there are other studies showing that nitric oxide induces HO-1 enzymes in vascular and endothelial cells [34, 35]. The abovementioned literature data are in accord with results of the present study.

In the present study, administration of L-arginine to both I/R rats and ischemic rats significantly increased HO-1 concentrations. Increased HO-1 activities, increased endogenous anti-oxidant enzyme concentrations, such as SOD, and increased inhibition of enzymes, such as MDA and MPO, are among the deficiencies of this study. This study failed to measure pancreatic blood flow as a measure of induction of NO synthesis by administration of L-arginine, a display that reverses the disorder in the microcirculation.

MPO is thought to be a marker of neutrophil activity. The release of MPO from neutrophils, which accumulate in injured tissues, triggers the formation of oxygen radicals and causes inflammation to intensify [36, 37]. Schanaider and colleagues reported that MPO activity correlates with the severity of pancreatitis in pro-inflammatory cytokine levels, such as TNF-alpha and IL-1beta, in an I/R induced acute pancreatitis study [37]. In other experimental pancreatitis model studies, there were also increased MPO concentrations, MPO enzyme is a marker of neutrophil activity, which plays an important role in tissue damage [38]. It is also thought that lipid peroxidation is the most important mechanism of the pathophysiological process of tissue damage caused by free oxygen radicals. MDA, the last product of fatty acid peroxidation, is used to determine the severity of tissue damage. Previous studies have shown a correlation between severity of acute pancreatitis and MDA levels. MDA levels in severe pancreatitis were found to be significantly higher than in mild pancreatitis [37]. In this study, MPO and MDA enzyme activities in I/R and only ischemic rats were found to be significantly higher than those in the sham group, in accord with the studies mentioned above. In addition, concentrations of these enzymes in the saline+I/R group were significantly higher than those in the ischemic group alone. These results have shown us that acute pancreatitis MPO and MDA enzyme activities are important in determining the severity of tissue damage. Administration of L-arginine in rats in the study group with a reduction of MPO and MDA concentrations was a sign of inhibition of oxidative stress in microcirculation disorder.

Under normal circumstances, oxygen used by the metabolism also forms reactive oxygen products and is neutralized by the anti-oxidants in the tissues. However, the balance between oxidants and anti-oxidants in the event of I/R-related damage is degraded in favor of oxidants [22]. SOD, an endogenous antioxidant, is the first acting enzyme in the antioxidant system. It is associated with increased activity of reduced levels of free oxygen radicals. Especially in experimental studies, molecules mimicking SOD activity have been shown to reduce pancreatic damage, MPO, and MDA levels, thus mortality [24]. In this study, an increase in SOD activity was found to neutralize I/R-associated anti-oxidants. In addition, administration of L-arginine to this group of rats suggests that the highest SOD levels are seen and that L-arginine

induces tissue damage-reducing effects by inducing the antioxidant system of the metabolism.

Histopathological changes, such as pancreatic edema, acinar cell degeneration, necrosis, and inflammation levels, were found to be the highest score in the I/R+saline group. The tissue damage-reducing effects of L-arginine administration over HO-1 activity were also shown histopathologically in this presented study. It has also been shown histologically that the reduction of tissue damage by HO-1 enzyme activity is not only due to anti-inflammatory effects, but also occurs intensively in anti-protective and micro-circulatory corrective effects. These results may be a promising therapeutic approach to reduce HO-1 enzyme activity, especially edema and inflammation in pancreatic tissue, which may be induced by oxidative stress and micro-vascular circulation in the early period of acute pancreatitis and induced by L-arginine administration.

As a result, oxidative stress caused by microcirculation disorders resulting from acute pancreatitis due to I/R injury causes tissue damage. Results showed that the administration of acute pancreatitis L-arginine due to I/R injury positively effects the mechanisms of damage reduction by inducing HO-1 enzyme activity. However, to investigate the relationship between these mechanisms and L-arginine, in more detail, clinical and experimental studies are needed.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kursat Dikmen, Department of General Surgery, Medical Faculty, Gazi University, Ankara 06520, Turkey. Tel: +903122-025727; Fax: +903122213202; E-mail: kursatdikmen66@gmail.com

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