RESEARCH ARTICLE

Antioxidant, anti-inflammatory and hepatoprotective effects of silymarin on hepatic dysfunction induced by sodium nitrite

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ABSTRACT. ***Purpose:*** **Sodium nitrite, a food additive that is used as a color fixative and preservative for meats and fish, has been reported to have adverse health effects due to increased oxidative stress that could be harmful to different organs including the liver. Meanwhile, silymarin protects against hepatotoxicity caused by a variety of agents, on account of its antioxidative and anti-inflammatory effects. We therefore examined the impact of dietary silymarin on sodium nitrite-induced liver damage in rats.** ***Methods:*** **Fifty adult male Sprague-Dawley rats received 80 mg/kg sodium nitrite in the presence or absence of silymarin (10 and 25 mg/kg). Hepatic proinflammatory cytokines (TNF-α and IL-1β), hepatic fibrosis marker (MCP-1 and TGF-β1), mitochondrial activity marker (cytochrome C oxidase) and c-reactive protein (CRP) levels were measured. Hepatic apoptosis was assessed through determination of caspase-3 activity and DNA fragmentation.** ***Results:*** **We found that oral sodium nitrite enhanced oxidative stress with subsequent increases in TNF-α (2-fold), IL-1β (4-fold), MCP-1 (4-fold), TGF-β1 (3-fold) and CRP (4-fold). In addition, sodium nitrite brings about reduced cytochrome C oxidase and enhanced caspase-3 activity and DNA fragmentation. Daily treatment with silymarin markedly ameliorated all these effects.** ***Conclusions:*** **Silymarin ameliorated the impairment of hepatic function in rats that had ingested sodium nitrite. Silymarn possesses antioxidant, anti-inflammatory, antifibrotic and anti-apoptotic effects.**

Key words: caspase-3, CRP, cytochrome C oxidase, DNA fragmentation, IL-1β, MCP-1, sodium nitrite, TGF-β1 and TNF-α

Humans are continuously exposed to different kinds of chemicals such as food additives. Many of these additives have been increasingly recognized as potentially hazardous to human health. Sodium nitrite is a food additive that is used as a color fixative and preservative for meats and fish [1]. Sodium nitrite is well known for its role in inhibiting the growth of *Clostridium botulinum* spores in refrigerated meats [2]. While sodium nitrite will prevent the growth of bacteria, in large amounts it can be toxic to animals, including humans. The cytotoxicity and detrimental effects of nitrite can be attributed to its oxidative properties. The reactive nitrogen species that are produced by exposure to nitrite have many toxic effects including hepatotoxicity, nephrotoxicity and dysregulation of inflammatory responses and tissue injury [3, 4]. We have previously reported that sodium nitrite caused hepatic impairment through several mechanisms, including oxidative stress, enhanced monocyte chemotactic protein (MCP)-1, deactivation of mitochondrial function, and DNA fragmentation [5].

Recent trends in controlling and treating diseases have tended to promote a more "natural" approach to food, as the diet plays an essential role in protecting the body against the development of certain conditions. An important natural product is silymarin. Silymarin is a polyphenolic, flavonoid antioxidant isolated from the fruits and seeds of the milk thistle, *Silybum marianum* (Asteraceae). Silymarin protects against hepatotoxicity caused by a variety of agents [6, 7]. Moreover, silymarin possesses a number of additional biological effects, such as an anti-oxidative activity [8], an anti-inflammatory effect [9], and it inhibits tumor necrosis factor (TNF)-α expression [10].

Recent understanding of the molecular events associated with increased level of sodium nitrite has focused on oxidative stress in different body organs [11]. Less attention has been paid to using natural treatments such as silymarin. This study is therefore designed to examine the impact of dietary silymarin on sodium nitrite-induced liver damage in rats. In particular, we wanted to investigate the effect of silymarin on oxidative stress, inflammation, mitochondrial activity, and cell death induced by sodium nitrite in rats.

METHODS

Animals and treatment protocols

The animal treatment protocol was approved by ethics committee of the Faculty of Pharmacy, University of Mansoura. Male Sprague Dawley rats weighing 180-200 g were used. All animals in the study were maintained under standard conditions of temperature, about 25°C, with a regular

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12 h light/12 h dark cycle, and allowed free access to food and water. All rats received treatment via oral gavage. Rats were classified into the following groups with 10 rats in each group:

Control group. Rats received the standard diet without any treatment and served as negative control group throughout the study.

Silymarin-treated control group. Rats received a daily standard diet and supplemented orally with 25 mg/kg silymarin (Sigma-Aldrich, St. Louis, MO, USA) for 12 weeks.

Sodium nitrite group. Rats received the standard diet and given sodium nitrite (Sigma-Aldrich) orally at a dose of 80 mg/kg body weight, daily for 12 weeks.

Silymarin-treated group (10 mg/kg). Rats received the standard diet, supplemented with 10 mg/kg silymarin followed by 80 mg/kg sodium nitrite administered, daily for 12 weeks.

Silymarin-treated group (25 mg/kg). Rats received the standard diet, supplemented with 25 mg/kg silymarin, followed by 80 mg/kg sodium nitrite, daily for 12 weeks.

The doses and time course of experiments used for sodium nitrite and silymarin in this study were in the range used in other studies applied for the same animal species [5, 12, 13]. In addition, the dose was determined after appropriate preliminary experiments.

Animal sacrifice and collection of samples

The animals were sacrificed by decapitation. Rat trunk blood was collected and centrifuged at 3,000 rpm for five minutes and serum samples were separated and stored at -80°C. Rat livers were removed, cleaned with ice-cold saline, weighed and chilled over crushed ice. A piece of the liver was homogenized in a 10-fold volume of ice-cold sodium, potassium phosphate buffer (0.01 M, pH 7.4) containing1.15% KCl. The homogenates were centrifuged at 600 g at 4°C for 10 minutes. The supernatant, referred to as homogenate, was stored at -80°C until used.

Measuring liver function

Serum alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and alanine aminotransferase (ALT) activities, as well as serum albumin and bilirubin concentrations, were measured by standard methodologies using commercially available kits provided by Biodiagnostic Company (Giza, Egypt).

Assessment of oxidative stress

Oxidative stress was estimated using the following parameters:

Hepatic malondialdhyde (MDA) concentration was measured using thiobarbituric acid as described previously by our group [14, 15]. In brief, after precipitation of proteins by trichloroacetic acid, thiobarbituric acid reacts with MDA to form thiobarbituric acid-reactive substance that is measured at 532 nm.

Hepatic glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-R) activities were measured using commercially available kits provided by Bio-Diagnostic Company, Giza, Egypt.

ELISA determination

The levels of biochemical parameters in liver homogenate were measured by ELISA assay using a commercially available MCP-1, TNF-α, IL-1β, transforming growth factor (TGF)-β1 and C-reactive Protein (CRP) ELISA kits (eBioscience Inc., San Diego, CA, USA), in accordance with the manufacturer's instructions.

Determination of hepatic mitochondrial function

Hepatic mitochondrial function was measured via determination of hepatic cytochrome C oxidase using a commercially available kit (Sigma-Aldrich). It is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome C by cytochrome C oxidase.

Estimation of apoptotic markers

Caspase-3 activity assay. Caspase-3 enzyme activity was measured colorimetrically using commercially available kits (GenScript, Piscataway, NJ, USA), following the manufacture's instructions.

DNA fragmentation assay. The DNA fragmentation assay was conducted using the procedure of Gercel-Taylor [16]. Liver tissue homogenates were centrifuged at 13,000 $\times$ g at 4°C for 15 min to separate the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). Pellet and supernatant fractions were assayed for DNA content using a freshly prepared diphenylamine solution and the optical density was read at 600 nm. Results are expressed as percentage fragmented DNA using the following formula: percentage fragmented DNA = $T \times 100/(T + B)$.

Statistical analysis

The mean values $\pm$ standard error were used for quantitative variables. For comparison between two groups Student's t-test was used. Statistical computations were done on a personal computer using the computer software SPSS version 13 (Chicago, IL, USA). Statistical significance was predefined as $P \leq 0.05$.

RESULTS

Effect of silymarin on liver function

Administration of sodium nitrite to rats resulted in marked cellular, molecular and biochemical changes that included liver impairment, fibrosis, mitochondrial function impairment, inflammation and DNA degradation. Liver function was measured by assessment of serum GGT, ALT, ALP, bilirubin and albumin. These parameters were utilized as sensors for the extent of the hepatoprotection effects of silymarin. As shown in *table 1*, sodium nitrite caused significant increases in serum ALT, ALP and GGT activities and bilirubin concentrations as compared with the control groups ($p<0.05$). In addition, sodium nitrite caused significant decreases in serum concentrations of albumin as compared with the control rats ($p<0.05$). Treatment with silymarin daily for 12 weeks resulted in significant, dose-dependent improvements in liver function markers in the sodium nitrite group and did not affect the control group.

Table 1
Liver function tests of different rat groups (mean ± SE).

	Control (n = 10)	C + Silymarin (25 mg/kg) (n = 10)	SN (80 mg/kg) (n = 10)	SN + Silymarin (10 mg/kg) (n = 10)	SN + Silymarin (25 mg/kg) (n = 10)
Serum ALT (U/L)	24.3 ± 1.9	25.4 ± 0.98	38.9 ± 2.4*	31.6 ± 2.9*#	25.4 ± 1.9#$
Serum ALP (U/L)	81.2 ± 1.2	80.4 ± 1.5	95.6 ± 3.2*	92.1 ± 2.8*	81.8 ± 3.1#$
Serum GGT (U/L)	42.7 ± 3.6	41.3 ± 2.9	53.6 ± 3.1*	51.9 ± 2.3*	44.7 ± 2.1#$
Serum albumin (g/dl)	4.6 ± 0.4	4.7 ± 0.3	3.2 ± 0.3*	3.6 ± 0.3*	3.8 ± 0.2*
Serum bilirubin (mg/dl)	0.67 ± 0.06	0.7 ± 0.05	1.8 ± 0.1*	1.2 ± 0.2*#	1.2 ± 0.1*#

C: control, SN: sodium nitrite, GGT: gamma glutamyltransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase.
* Significant difference as compared with the control groups at $p<0.05$.
# Significant difference as compared with the sodium nitrite group at $p<0.05$.
$ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.

Effect of silymarin on hepatic oxidative stress levels

Sodium nitrite resulted in significant increases in the hepatic levels of MDA, as well as significant decreases in hepatic activities of GSH-Px and GSH-R as compared with the control groups ($p<0.05$). However, treatment of rats with silymarin resulted in significant, dose-dependent reductions in MDA levels and significant increases in the activities of GSH-Px and GSH-R in the sodium nitrite group; the control group was not affected (*figure 1*).

Effect of silymarin on hepatic proinflammatory cytokines

As regards proinflammatory cytokines, we found significant increases in hepatic concentrations of TNF-α and IL-1β in the sodium nitrite group as compared with the control group ($p<0.05$). However, daily administration of sodium nitrite with silymarin for 12 weeks resulted in significant, dose-dependent reductions in hepatic levels of both TNF-α and IL-1β, as compared with the sodium nitrite group. Treatment with silymarin did not affect the control group (*figure 2*).

Effect of silymarin on hepatic acute inflammation markers

We found significant increases in hepatic concentrations of CRP in the sodium nitrite group as compared with the control group ($p<0.05$). Rats in the sodium nitrite group treated with silymarin showed a significant, dose-dependent reduction in hepatic levels of CRP as compared with the sodium nitrite only group. Treatment with silymarin did not affect the control group (*figure 3*). However, levels of CRP in rats treated with both doses of silymarin were still significantly higher than those of the control group ($p<0.05$).

Effect of silymarin on hepatic fibrosis marker

As shown in *figure 4*, we found a significant increase in hepatic MCP-1 and TGF-β1 in rats that received sodium nitrite as compared with the control rats ($p<0.05$). Treatment of sodium nitrite rats with silymarin resulted in a significant, dose-dependent reduction in hepatic MCP-1 and TGF-β1, as compared with rats that receive sodium nitrite only, and levels of MCP-1 and TGF-β1 in rats treated with both doses of silymarin were significantly higher than those of the control group. However, treatment with silymarin did not affect the control group.

Effect of silymarin on hepatic mitochondrial activity

As shown in *figure 5*, we found a 42% reduction in hepatic cytochrome C oxidase in rats that received sodium nitrite as compared with the control rats. Treatment with 25 mg/kg silymarin only restored cytochrome C oxidase activity in the sodium nitrite group; it did not affect the control group.

Effect of silymarin on hepatic apoptotic markers

Figure 6 shows that sodium nitrite caused a significant increase in the percentage of hepatic DNA fragmentation and hepatic caspase-3 activity as compared with the control group ($p<0.05$). Treatment with 25 mg/kg silymarin only showed significant reductions in hepatic caspase-3 activity and DNA fragmentation. However, the levels of DNA fragmentation and caspase-3 in the rats, simultaneously treated with sodium nitrite and both doses of silymarin, were still significantly higher than those of the control group ($p<0.05$).

DISCUSSION

The main findings of the current study are that treatment with silymarin results in a dose-dependent amelioration of the impairment of hepatic function in rats that had received sodium nitrite. This is thought to have occurred via multiple mechanisms including: (1) a reduction in sodium nitrite-induced oxidative stress, as indicated by reduced hepatic MDA levels and restored activity of hepatic GSH-R and GSH-Px; (2) a blocking of sodium nitrite-induced increases in hepatic proinflammatory cytokines such as TNF-α and IL-1β; (3) a reduction of sodium nitrite-induced increases in acute inflammation markers such as CRP; (4) a blocking of sodium nitrite-induced increases in hepatic fibrosis markers such as MCP-1 and TGF-1β; (5) inhibition of sodium nitrite-induced deactivation of mitochondrial function as indicated by restoration of cytochrome C oxidase activity; and (6) a reduction in sodium nitrite-induced activation of hepatic caspase-3 and of the increases in the percentage of DNA fragmentation. The mechanisms of action are summarized in *figure 7*.

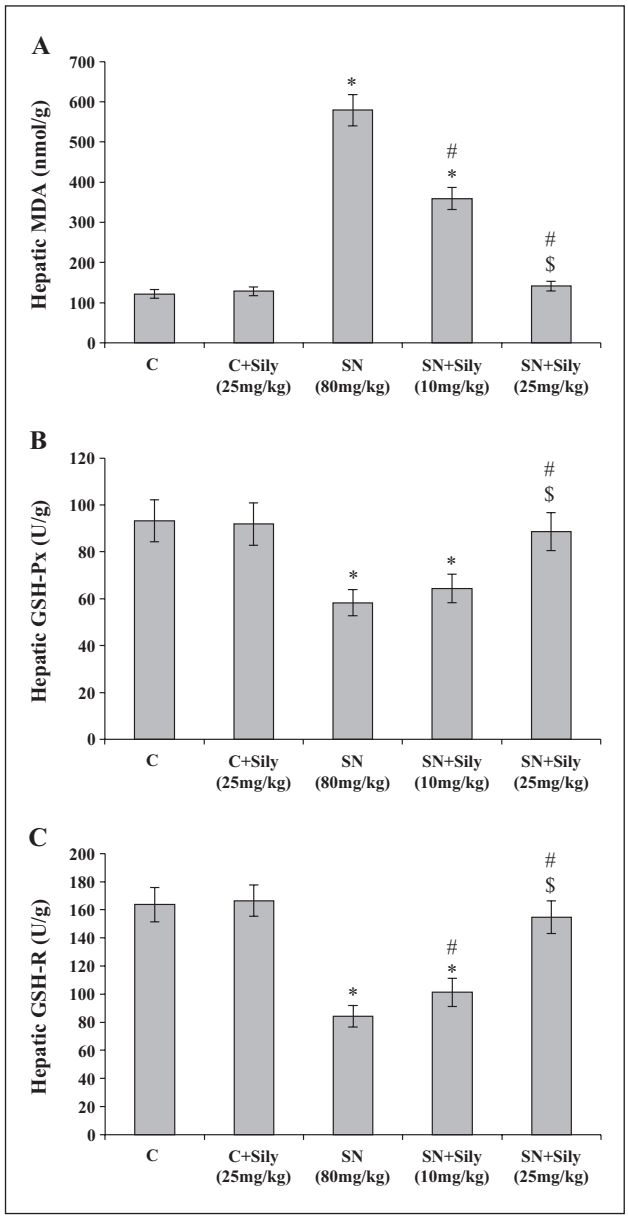

Figure 1
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks on hepatic MDA **(A)**, GSH-Px **(B)** and GSH-R **(C)**.
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$. $ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.
C, control; SN, sodium nitrite; MDA, malondialdehyde; GSH-Px, glutathione peroxidase; GSH-R, glutathione reductase.

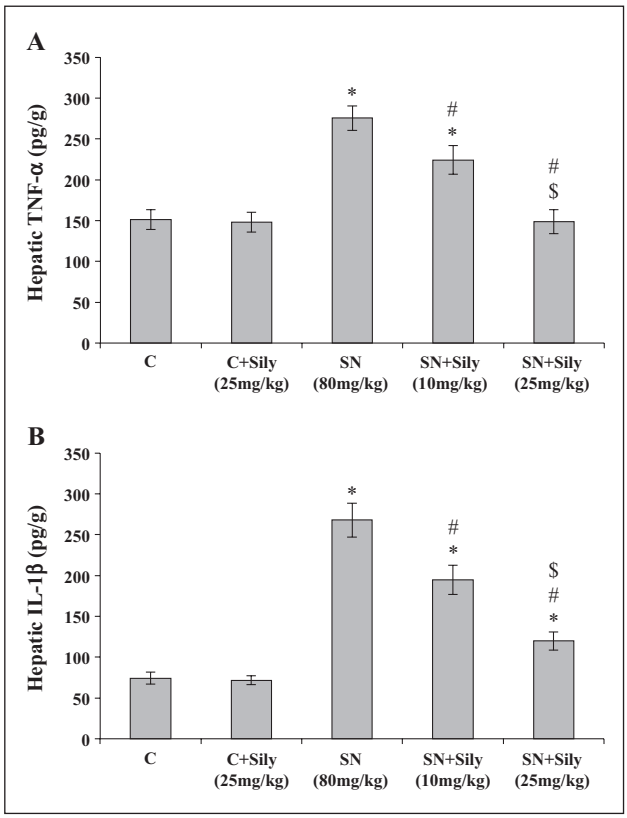

Figure 2
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks on hepatic TNF-α (A) and IL-1β (B).
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$. $ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.
C, control; SN, sodium nitrite; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

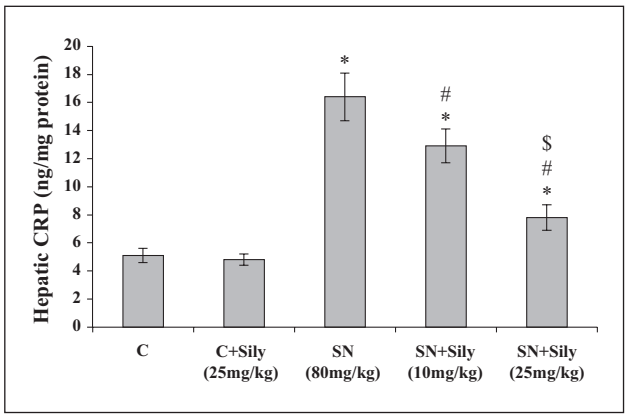

Figure 3
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks on hepatic CRP.
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$. $ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.
C, control; SN, sodium nitrite; CRP, C reactive protein.

Nitrite is an important antimicrobial additive for food products. Nitrite in meat greatly delays the development of *botulinum* toxin, develops cured meat flavor and color, retards the development of rancidity during storage, inhibits the development of warmed-over flavor and preserves the flavors of spice and smoke [17]. Despite the enormous effort over the past few decades to limit dietary nitrite consumption because of its potential to form carcinogenic N-nitrosamines, to date there are no conclusive data to suggest that dietary sources of nitrite may be unsafe. However, the negative connotations of nitrite remain and have led governments to regulate and restrict levels in food and drinking water, particularly in cured and processed meats. We found that administration of sodium nitrite alone for 12 weeks resulted in liver function impairment manifesting as significant changes in all biochemical parameters tested. These results bear out the hepatic impairment effect of sodium nitrite in rats, which is in consistent with previous studies [1, 5]. Sodium nitrite caused oxidative damage

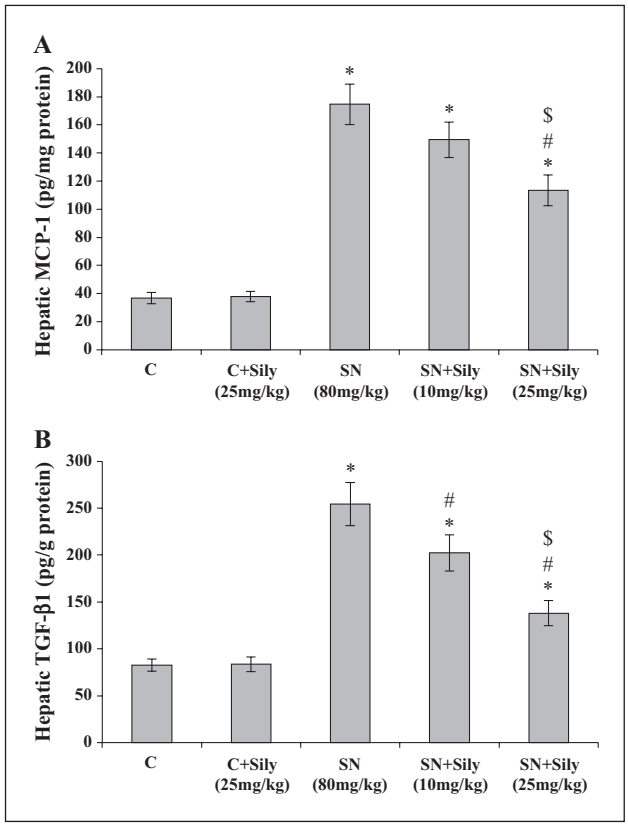

Figure 4
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks on hepatic MCP-1 (A) and TGF-β1 (B).
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$. $ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.
C, control; SN, sodium nitrite; TGF-β1, transforming growth factor-β1; MCP-1, monocyte chemotactic protein-1.

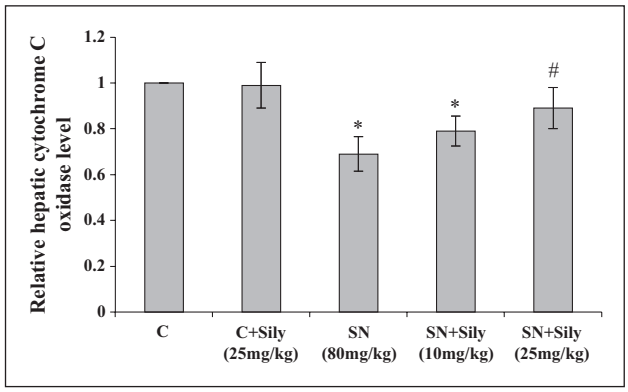

Figure 5
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks, on hepatic cytochrome C oxidase.
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$.
C, control; SN, sodium nitrite.

to cell membrane, liver tissue damage and inhibition of oxidative stress, resulting in the increased activity of liver enzymes and reduced albumin levels [5].

The relationship between diet and health has been recognized throughout recorded history. Disease prevention through eating habits and healthy preparation of foods

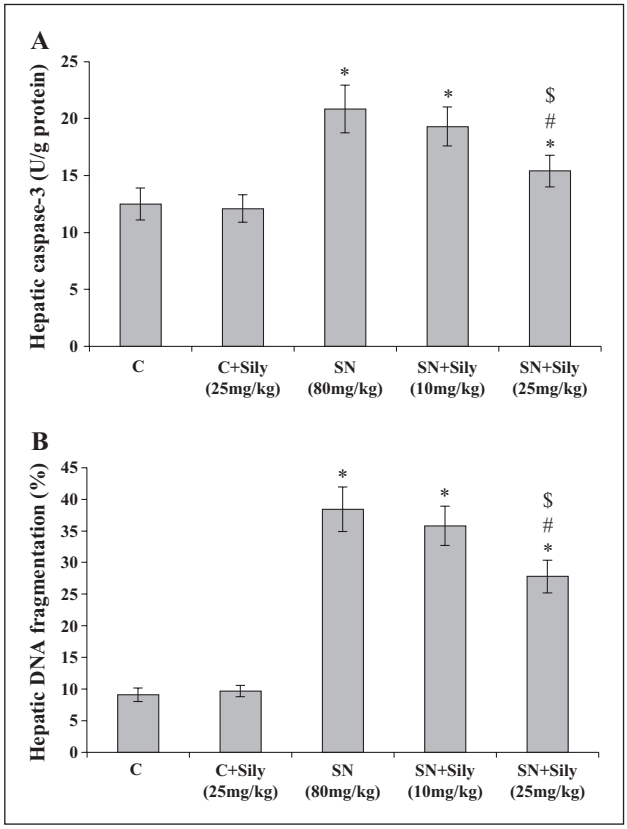

Figure 6
Effect of sodium nitrite (SN, 80 mg/kg/day) alone and in combination with, silymarin (Sily, 10 and 25 mg/kg/day) for 12 weeks on hepatic caspase-3 (A) and DNA fragmentation (B).
* Significant difference as compared with the control groups at $p<0.05$. # Significant difference as compared with the sodium nitrite group at $p<0.05$. $ Significant difference as compared with sodium nitrite+silymarin (10 mg/kg) group at $p<0.05$.
C, control; SN, sodium nitrite.

has been discussed in religious and civil writings for thousands of years. We therefore tried to investigate the role of silymarin in a sodium nitrite rat model. Daily treatment with silymarin for 12 weeks ameliorates the altered liver enzymes, bilirubin and albumin in sodium nitrite groups, but did not affect the control group. Silymarin has a protective effect in many models of hepatotoxicity such as ethanol-induced hepatotoxicity [18], carbon tetrachloride-induced liver injury [19], paraquat-induced hepatotoxicity [10], manganese-induced hepatic damage [20], doxorubicin-induced hepatotoxicity [21], paracetamol-induced hepatotoxicity [22], cisplatin-induced hepatotoxicity [23], and iron overload-induced hepatotoxicity [24]. To our knowledge, no study has yet investigated the potential beneficial effects of silymarin in preventing sodium nitrite-induced hepatic impairment.

Next, we tried to figure out the mechanism of the hepatoprotective effect of silymarin. Inside the body, the free radical species produced by exposure to nitrite is considered to be one of the most important causes of body tissues destruction, for example, lipid peroxidation, DNA lesions, enzyme inactivation and damage to other organs. The indicator of high oxidative stress, lipid peroxidation, can be attributed to the oxidative cytotoxicity of nitrite [25]. It has been reported previously that sodium nitrite and other food additives may react with amines in foods in the stomach producing nitrosamines and free radicals. Such products may increase lipid peroxidation, which can be harmful to

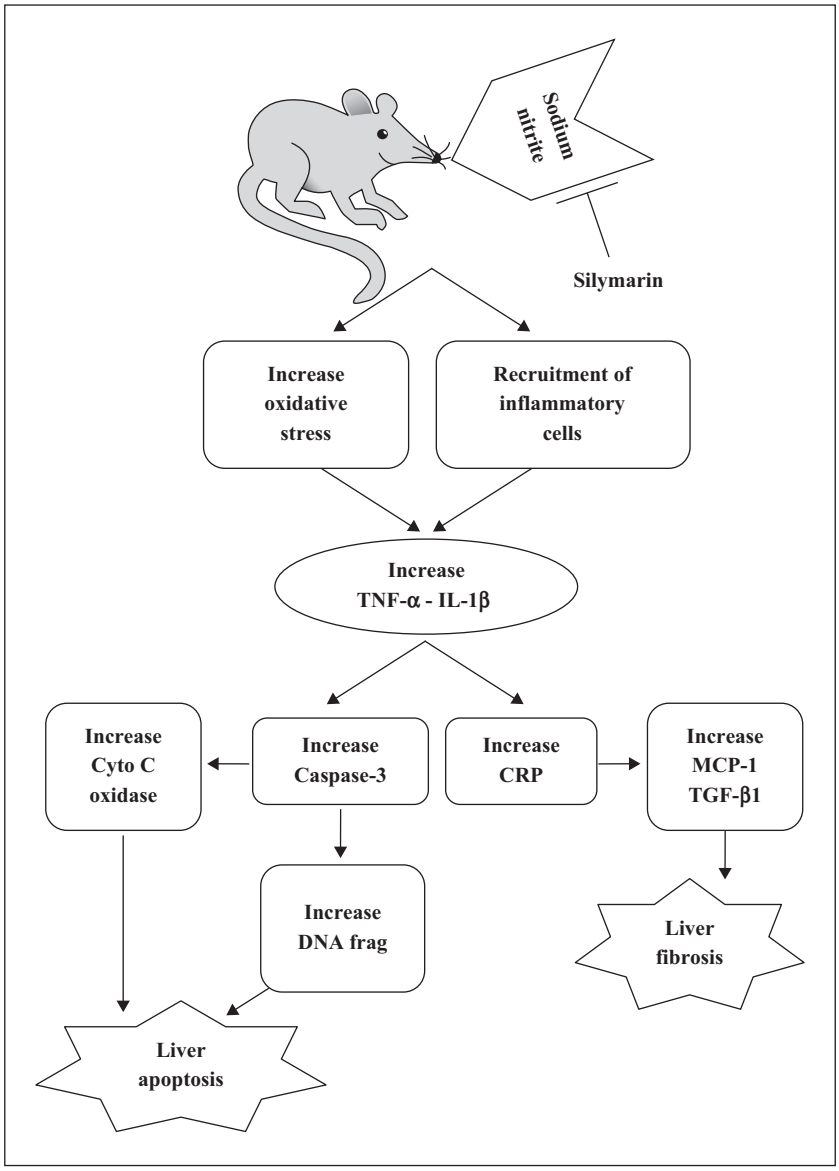

Figure 7
Schematic representation of the mechanism of the hepatoprotective action of silymarin against sodium nitrite-induced liver damage.

different organs including the liver [11, 26]. We found significant increases in MDA and significant decreases in antioxidant activity (GSH-R and GSH-Px) in hepatic homogenates, which were consistent with previous studies [1, 5]. All these effects were blocked by silymarin. Many studies have shown that silymarin is capable of protecting liver cells directly by stabilizing membrane permeability by inhibiting lipid peroxidation and preventing liver glutathione depletion [27].

We studied the effect of sodium nitrite on proinflammatory cytokines. We observed significant increases in hepatic TNF-α and IL-1β in the sodium nitrite group compared with the control group. Similarly, Sun *et al.*, 2006, found that IL-1β, IL-6 and TNF-α increased in human gastric cells following exposure to sodium nitrite [28]. This perhaps may be explained by the increase in oxidative stress and activation of proinflammatory cytokines caused by the sodium nitrite. Many stimuli have been reported to upregulate pro-inflammatory cytokines, including TNF-α, through oxidative stress and activation of NF-κB [29, 30]. In addition, sodium nitrite caused significant increases in hepatic CRP levels. CRP, an acute-phase reactant produced by the liver in response to inflammation, is synthesized by the liver in response to factors released by macrophages and adipocytes [31].

Next, we found significant increases in hepatic fibrosis markers, MCP-1 and TGF-β1 in sodium nitrite-treated rats. Vitaglione *et al.*, 2004, reported that fibrogenic mediators such as TGF-β1 and MCP-1 are responsible for a series of inflammatory and fibrotic process in liver injury [32-34]. The increases in inflammatory and fibrogenic markers were ameliorated by silymarin. However, NF-κB, which transactivates a number of downstream proinflammatory genes, was inhibited by silymarin [35]. Moreover, silymarin has a potent anti-fibrogenic action in the liver by reducing fibrogenic cytokine TGF-β1 expression in a model of liver fibrosis [36].

Moreover, the effect of sodium nitrite on mitochondrial activity, as demonstrated by cytochrome C oxidase, was also assessed in the present study. Mitochondrial cytochrome C oxidase, a copper-containing metalloenzyme, is the final electron acceptor in the mitochondrial electron transport chain and is required for aerobic ATP production. Cytochrome C oxidase transverses the inner

mitochondrial membrane, with portions protruding into the intermembrane space and the matrix. It catalyzes electron transfer from cytochrome C to molecular oxygen [37]. We found a significant decrease in the cytochrome C oxidase activity in rats that received oral sodium nitrite. It has been previously proposed that this down-regulation of cytochrome C oxidase activity may be linked to reactive oxygen species [38]. In addition, the decrease in cytochrome C oxidase activity was observed during oxidative stress and apoptosis [38, 39]. Of note, we found a significant increase in oxidative stress that was accompanied by a significant reduction in cytochrome C oxidase in rats that had received sodium nitrite. However, we also demonstrated, for the first time, the significance of using silymarin in restoring hepatic cytochrome C oxidase activity in rats.

Caspases are a family of cysteine proteases activated during apoptosis [40]. However, reactive oxygen species are believed to cause genetic oxidation and damage to DNA and other macromolecules [41]. Our result demonstrated that administration of sodium nitrite resulted in a significant increase in caspase-3 activity and DNA fragmentation, which are blocked by silymarin. However, there are no prior studies investigating the effects of silymarin on TNF-α, IL-β, MCP-1, TGF-β1, caspase-3, and DNA fragmentation in the liver of sodium nitrite-treated rats with which to compare this study.

Disclosure. *Financial support: none. Conflict of interest: none.*

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