

Cod liver oil in sodium nitrite induced hepatic injury: Does it have a potential protective effect?

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Objectives: Exposure to sodium nitrites, a food additive, at high levels has been reported to produce reactive nitrogen and oxygen species that cause dysregulation of inflammatory responses and tissue injury. In this work, we examined the impact of dietary cod liver oil on sodium nitrite-induced inflammation in rats.

Methods: Thirty-two adult male Sprague-Dawley rats were treated with 80 mg/kg sodium nitrite in presence/absence of 5 ml/kg cod liver oil. Liver sections were stained with hematoxylin/eosin. We measured hepatic tumor necrosis factor (TNF)- α , interleukin-1 beta (IL)-1 β , C-reactive protein (CRP), transforming growth factor (TGF)- β 1, and caspase-3.

Results: Cod liver oil reduced sodium nitrite-induced hepatocyte damage. In addition, cod liver oil results in reduction of hepatic TNF- α , IL-1 β , CRP, TGF- β 1, and caspase-3 when compared with the sodium nitrite group.

Discussion: Cod liver oil ameliorates sodium nitrite-induced hepatic injury via multiple mechanisms including blocking sodium nitrite-induced elevation of inflammatory cytokines, fibrosis mediators, and apoptosis markers.

Keywords: Caspase-3, C-reactive protein (CRP), Interleukin (IL)-1 β , Transforming growth factor (TGF)- β 1, and Tumor necrosis factor (TNF)- α

Introduction

Humans are continuously exposed to different kinds of chemicals such as food additives. Many of these additives have been increasingly recognized as potentially hazardous for human health. One important food additive is sodium nitrite. It is well known for its role in inhibiting the growth of *Clostridium botulinum* spores in refrigerated meats by inhibiting iron-sulfur clusters essential to energy metabolism.¹ Moreover, sodium nitrite is able to effectively delay the development of oxidative rancidity by reacting with heme proteins and metal ions and chelating free radicals, terminating the cycle of lipid oxidation that leads to rancidity.² However, exposure to nitrites at levels above health-based risk, LD₅₀ 71 mg/kg in humans, has been reported to produce cytotoxic and weak carcinogenic effects.^{3,4} Reactive nitrogen species produced by exposure to nitrite have many toxic effects including hepatotoxicity, nephrotoxicity, and dysregulation of inflammatory responses and tissue injury.^{5,6}

Recent trends in controlling and treating diseases tend to favor natural compounds, as the human diet is essential in protecting the body against the development of diseases. One of these natural products is cod liver oil, derived from the liver of genus *Gadus* of demersal fish in the *Gadidae* family. It has been used for medicinal purposes for centuries. One dose of cod liver oil supplement (5 ml) contains 500 mg vitamin A, 10 mg vitamin D, and 10 mg vitamin E as well as 1.2 g of ω -3 fatty acids.⁷ Cod liver oil has many favorable biological properties such as antiinflammatory, antioxidant, and antifibrosis properties.⁸⁻¹⁰

Recent understanding of the molecular events of increased levels of sodium nitrite has focused researchers' attention on oxidative stress in different body organs.^{8,11} Less attention has been paid to other effects like inflammation. Therefore, this study is designed to examine the impact of dietary cod liver oil on chronic exposure to oral sodium nitrite and its subsequent induced liver inflammation in rats.

Materials and methods

Animals and their treatment outlines

The animal protocol was approved by the ethical committee of the Faculty of Pharmacy, University of

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Mansoura (protocol code no. 2012–32). Male Sprague-Dawley rats weighing 120–140 g were used. All animals in the study were maintained under standard conditions of temperature, about 25°C, with regular 12 hours light/12 hours dark cycle and allowed free access to food and water. Rats were classified into the following groups with eight rats in each group:

Control group: Rats received the standard diet without any treatment.

Cod liver oil control group: Rats received 5 ml/kg cod liver oil (Silver Seas Co., El-Obour, Egypt) daily by oral gavage for 12 weeks.

Sodium nitrite group: Rats were given daily sodium nitrite (Sigma-Aldrich Co, St. Louis, MO, USA) at a dose of 80 mg/kg body weight dissolved in phosphate buffer solution, pH7.4, by oral gavage for 12 weeks.

Cod liver oil treated group: Rats received 5 ml/kg cod liver oil. After 60 minutes rats were treated with 80 mg/kg sodium nitrite dissolved in phosphate buffer solution daily for 12 weeks.

The doses and time course of experiments used for sodium nitrite and cod liver oil in this study were in the range of those used in other studies in the same animal species.^{8,12–14} 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 3000 rpm for 5 minutes and serum samples were separated and stored at –80° C. Rat livers were removed, cleaned with ice-cold saline, weighed, and chilled on 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) containing 1.15% (w/v) 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 till used.

Assessment of liver function tests

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

Assessment of oxidative stress

Oxidative stress was estimated in the liver homogenates through the following parameters:

Superoxide anion concentration was measured by the nitroblue tetrazolium method.¹⁵ It depends on the ability of the superoxide anion to reduce nitroblue tetrazolium to an insoluble formazan that can be measured at 560 nm.

Superoxide dismutase (SOD) activity was determined using the phenazine methosulfate method,¹⁶

in which the ability of the enzyme to inhibit the phenazine methosulfate-mediated reaction of nitroblue tetrazolium dye is measured as an indication of the activity of SOD.

ELISA determination

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

Determination of caspase-3 activity assay

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

Morphologic analysis of hepatic tissue

The liver was cut, fixed in 10% (v/v) buffered formalin and embedded in paraffin. Five micrometer thickness sections were cut and stained with Mayer's hematoxylin and eosin for examination of cell structure by light microscopy. Hepatic specimens were anonymously coded and examined in a masked manner. The morphologic changes were photographed using a digital camera-aided computer system (Nikon Digital Camera, Tokyo, Japan).

Immunohistochemistry

Immunohistochemical analyses were performed on 5- μ m-thick paraffin sections cut from a paraffin block of liver. Sections were incubated with monoclonal anti-TNF- α (eBioscience Inc., San Diego, CA, USA) at 4°C. Slides were counterstained with hematoxylin and examined using a Nikon Digital Camera.

Statistical analysis

For descriptive statistics of quantitative variables, the mean \pm standard error was used. Normality of the sample distribution of each continuous variable was tested with the Kolmogorov–Smirnov test. One-way analysis of variance was used to compare means between groups. If differences existed among the means, a *post hoc* Bonferroni correction 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

Hepatoprotective effects of cod liver oil

An estimation of liver enzymes in serum was conducted to evaluate the hepatoprotective effects of cod liver oil by measuring the serum activities of ALT, ALP, and GGT (Fig. 1A). Rats treated with sodium

nitrite showed significant elevations in serum ALT, ALP, and GGT as compared with the control group. In parallel, liver sections stained with H&E showed fibrosis and breakdown of hepatic tissues (Fig. 1B). Treatment with cod liver oil reversed all these effects in the sodium nitrite group without affecting the control group.

Effect of cod liver oil on oxidative stress

We examined the effect of sodium nitrite on oxidative stress status in hepatic homogenates and found a significant increase in the hepatic levels of superoxide anion that was associated with about 46% reduction in SOD activity as compared with the control group ($P < 0.05$). Treatment with cod liver oil (5 ml/kg body weight, po) daily for 12 weeks significantly blocked these effects in the sodium nitrite group but did not affect the control group (Fig. 2A and B).

Effect of cod liver oil on hepatic proinflammatory cytokines

Treatment of rats with sodium nitrite was accompanied by marked cellular, molecular and

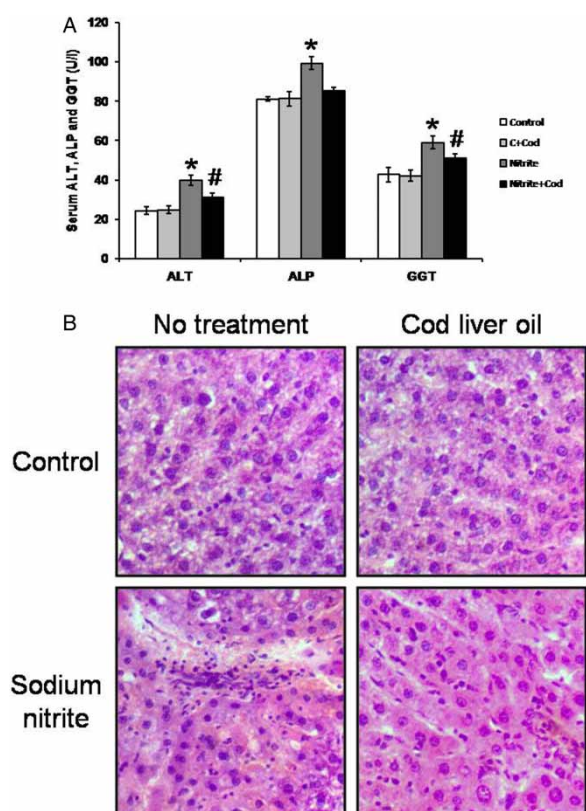


Figure 1 Effect of sodium nitrite (Nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on serum alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) (A) as well as liver sections stained with H/E (B). *Significant difference as compared with the rest of the groups at $P < 0.05$. #Significant difference as compared with the control group at $P < 0.05$.

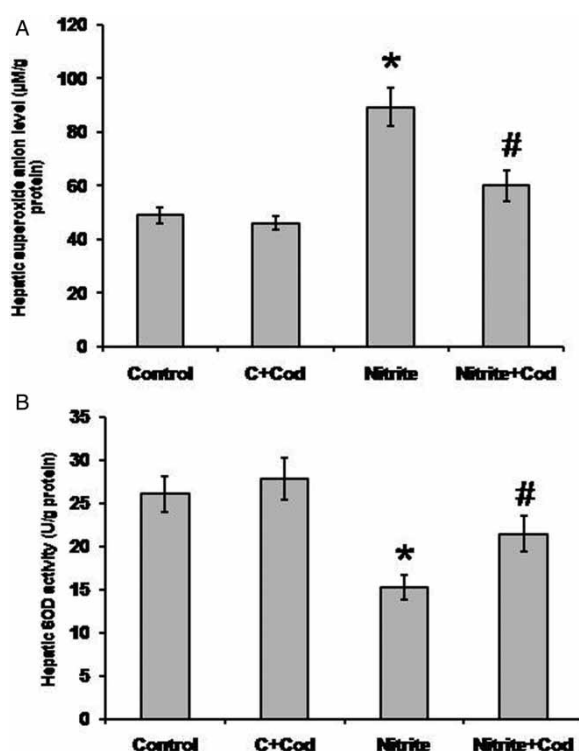


Figure 2 Effect of sodium nitrite (nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on hepatic superoxide anion concentration (A) and superoxide dismutase (SOD) activity (B). *Significant difference as compared with the rest of the groups at $P < 0.05$. #Significant difference as compared with the control group at $P < 0.05$.

biochemical changes that included liver impairment, fibrosis, mitochondrial function impairment, inflammation, and DNA degradation. Regarding proinflammatory cytokines, we found significant elevations in hepatic concentration of TNF- α (275.6 ± 15.1 pg/g) and IL-1 β (267.9 ± 20.9 pg/g) in the sodium nitrite group as compared with the control group (151.13 ± 12.1 and 74.1 ± 7.3 pg/g, respectively). However, treatment of sodium nitrite-dosed rats with cod liver oil showed significant reductions in hepatic level of both TNF- α and IL-1 β (196.1 ± 12.3 and 186.8 ± 16.7 pg/g, respectively) as compared with the sodium nitrite group, but still significantly higher than the control group ($P < 0.05$) (Fig. 3A and C). Treatment with cod liver oil did not affect the control group. In addition, rat liver sections stained with anti-TNF- α showed elevated TNF- α content in hepatocytes after sodium nitrite exposure that was reduced by treatment with cod liver oil (Fig. 3B).

Effect of cod liver oil on hepatic acute inflammation markers

As shown in Fig. 4, we found significant elevation in hepatic concentration of CRP in the sodium nitrite group (16.4 ± 1.7 ng/mg) as compared with the control group (5.1 ± 0.43 ng/mg). Treatment of the sodium nitrite group with cod liver oil caused a

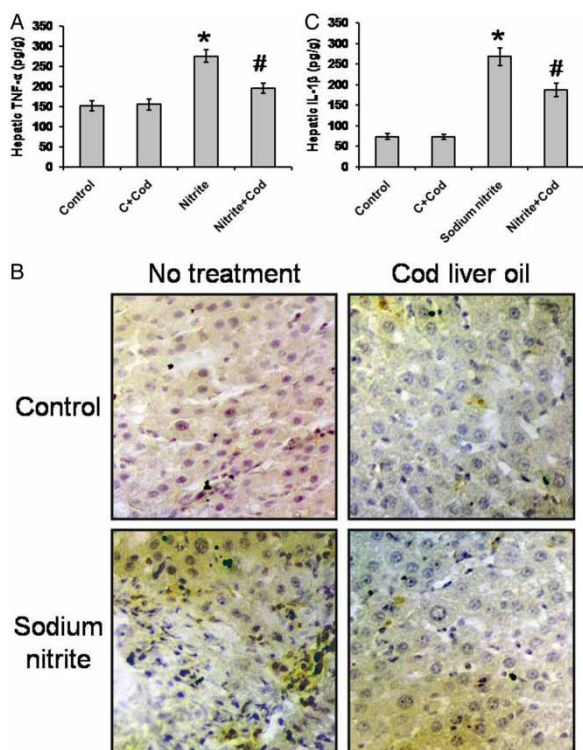


Figure 3 Effect of sodium nitrite (nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on hepatic tumor necrosis factor (TNF)-α (A) and interleukin (IL)-1β (C) as well as liver sections stained with monoclonal anti-TNF-α (B). *Significant difference as compared with the rest of the groups at $P < 0.05$. #Significant difference as compared with the control group at $P < 0.05$.

significant reduction in hepatic level of CRP (9.7 ± 0.8 ng/mg) as compared with the sodium nitrite alone group. However, the hepatic level of CRP in rats treated with both sodium nitrite and cod liver oil was still significantly higher than that in the control group ($P < 0.05$).

Effect of cod liver oil on hepatic fibrosis marker

As shown in Fig. 5, we found a significant increase in hepatic TGF-β1 in rats that received sodium nitrite

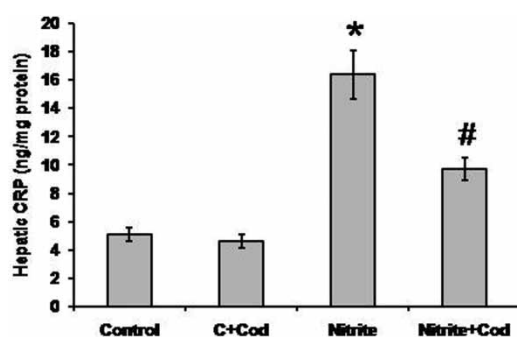


Figure 4 Effect of sodium nitrite (nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on hepatic C-reactive protein (CRP). *Significant difference as compared with the rest of the groups at $P < 0.05$. #Significant difference as compared with the control group at $P < 0.05$.

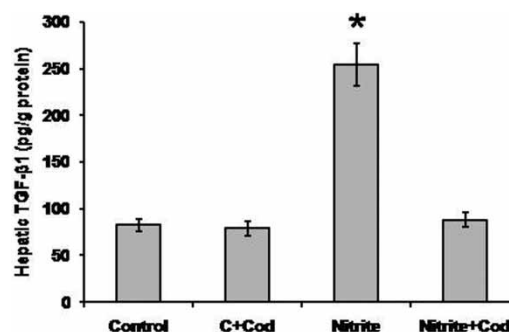


Figure 5 Effect of sodium nitrite (nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on hepatic transforming growth factor (TGF)-β1. *Significant difference as compared with the rest of the groups at $P < 0.05$.

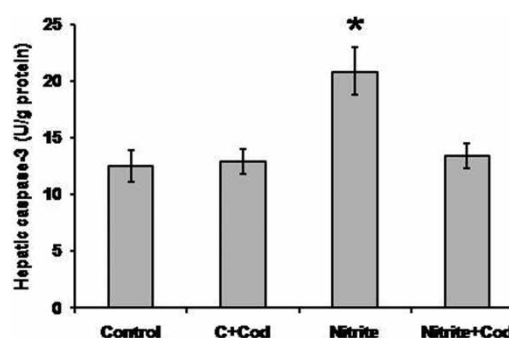


Figure 6 Effect of sodium nitrite (nitrite, 80 mg/kg/day) alone and its combination with cod liver oil (Cod, 5 ml/kg/day) for 12 weeks on hepatic caspase-3 activity. *Significant difference as compared with the rest of the groups at $P < 0.05$.

(254.5 ± 21.3 pg/g) as compared with the control rats (82.7 ± 6.6 pg/g). Treatment of sodium nitrite-dosed rats with cod liver oil blocked the increase in hepatic TGF-β1 (88.1 ± 7.9 pg/g) as compared with rats that received sodium nitrite only. In addition, treatment of the control group with cod liver oil did not affect the hepatic level of TGF-β1.

Effect of cod liver oil on hepatic apoptotic markers

Fig. 6 illustrates that sodium nitrite caused a significant increase in hepatic caspase-3 activity (20.9 ± 2.1 U/g) as compared with the control group (12.5 ± 1.1 U/g). Treatment with cod liver oil caused a significant reduction in hepatic caspase-3 activity (13.4 ± 0.9 U/g) in the sodium nitrite group but did not affect the control group.

Discussion

Nitrite is an important antimicrobial additive to food products that delays the development of botulinum toxin and retards the development of rancidity during storage.¹⁷ Despite the enormous effort over the past few decades to limit or even restrict dietary

nitrite consumption due to the potential to form carcinogenic N-nitrosamines, to date there are no conclusive data to indicate that dietary sources of nitrite may be unsafe. Chronic administration of sodium nitrite can result in liver impairment. Sodium nitrite caused oxidative damage to cell membrane, liver tissue damage and inhibition of oxidative stress resulting in elevating the activity of liver enzymes.^{8,11} Of note, we found that oral sodium nitrite resulted in a significant elevation of serum liver enzymes that was associated with elevated hepatic superoxide anion and reduced hepatic SOD activity.

We observed significant elevation in hepatic TNF- α and IL-1 β in the sodium nitrite group in comparison with the control group. Similarly, Sun *et al.*¹⁸ found that IL-1 β , IL-6, and TNF- α increased in human gastric cells after exposure to sodium nitrite. This perhaps may be explained by sodium nitrite elevation of oxidative stress and activation of proinflammatory cytokines. Many stimuli have been reported to upregulate proinflammatory cytokines, including TNF- α , through oxidative stress and activation of nuclear factor-kappa B (NF- κ B).^{19,20} In addition, we observed that sodium nitrite caused a significant increase in hepatic CRP level, an acute-phase reactant produced by the liver in response to inflammation. CRP is synthesized by the liver in response to factors released by macrophages and adipocytes.²¹

The relationship between diet and health has been recognized throughout recorded history. Therefore, we tried to investigate the effect of cod liver oil on the inflammation produced by sodium nitrite in rats. We found that treatment of rats with cod liver oil significantly ameliorated liver tissue damage as well as reducing the elevated hepatic levels of TNF- α , IL-1 β , and CRP in the sodium nitrite group. Cod liver oil was reported to have anti-inflammatory effects in different studies: pneumonia in mice²² and insulin resistance in humans.²³ The anti-inflammatory effects of cod liver oil can be attributed to its contents of ω -3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid (DHA), which have been reported to support anti-inflammatory responses in animals and humans.^{24–27} Several theories have been advocated to explain the ability of ω -3 fatty acids to antagonize inflammation that include competitive inhibition of conversion of arachidonate to proinflammatory lipid intermediates, generation of anti-inflammatory lipid mediators such as resolvins and protectins, and antagonism of NF- κ B signaling.^{28–30}

In addition, we found a significant elevation in hepatic TGF- β 1 in sodium nitrite-treated rats. However, TGF- β 1 is responsible for a series of inflammatory and fibrotic processes in liver injury.^{31,32} The increases in TGF- β 1 were ameliorated by cod liver

oil. This can be explained by ability of DHA to reduce fibrosis.^{33–35}

Caspases are a family of cysteine proteases activated during apoptosis.³⁶ Our results demonstrated that administration of sodium nitrite caused a significant elevation in caspase-3 activity, which was blocked by cod liver oil. However, there are no prior studies investigating the effects of cod liver oil on TNF- α , IL- β , CRP, TGF- β 1, and caspase-3 in livers of sodium nitrite-treated rats with which to compare this study.

Our study concluded that cod liver oil alleviates the hepatic injury induced by sodium nitrite via multiple mechanisms including (1) blocking elevation of hepatic proinflammatory cytokines such as TNF- α and IL-1 β ; (2) reducing the elevation of the acute inflammation marker, CRP; (3) blocking the elevation of the hepatic fibrosis marker, TGF- β 1; and (4) reducing the activation of the hepatic apoptotic enzyme, caspase-3.

Disclaimer statements

Contributors Iman O Sherif and Mohammed M Al-Gayyar contributed equally to this study.

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Conflicts of interest None.

Ethics approval The animal protocol was approved by ethical committee in Faculty of Pharmacy, University of Mansoura (protocol code no. 2012-32).

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