Effects of Vitamin C and Melatonin on Cysteamine-Induced Duodenal Ulcer in a Cholestatic Rat Model: A Controlled Experimental Study

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ABSTRACT

BACKGROUND: Superoxide dismutase (SOD) is one of the defense mechanisms against free radicals. Cysteamine is a cytotoxic agent, acting through generation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, and superoxide, and may decrease defense activity of SOD against ROS and induce duodenal ulcer. Melatonin is a suicidal antioxidant that has a protective effect against ROS and cytoprotective effect through inhibition of the decrease in SOD activity.

OBJECTIVES: The primary aim of this study was to assess the effects of pretreatment with vitamin C and melatonin on cysteamine-induced duodenal ulcer. Secondary aims were to compare the ulcerogenic effect of cysteamine and the antiulcer effects of vitamin C and melatonin.

METHODS: This study was performed in male Wistar rats (200–250 g) in 3 groups of equal size (n = 24): bile duct ligation–induced cholestasis (test), sham, and control groups. In the test and sham groups, laparotomy was performed under general anesthesia and the common bile duct was identified; in sham rats, the common bile duct was left in situ, but in test rats, the common bile duct was isolated and doubly ligated to induce cholestasis. Animals in each group were also divided into 4 equal subgroups (n = 6). These subgroups were treated with vitamin C plus cysteamine, melatonin plus cysteamine, cysteamine alone, and saline, respectively. All animals were euthanized via overdose of ether anesthesia 24 hours after the last injection of cysteamine or saline, and 0.5 mL of blood was collected from the heart ventricle. The duodenum was cut open, washed with saline, fixed, and prepared for calculation of ulcer index (Szabo method) and histopathologic assessment. SOD activity was measured using a branded enzyme kit.
RESULTS: In all 3 groups, animals treated with cysteamine had significantly increased mean (SE) ulcer index (test, 4.00 [0.10] vs 1.17 [0.30]; sham, 3.83 [0.16] vs 0.50 [0.22]; control, 3.67 [0.21] vs 0 [0]) and decreased SOD activity (test, 146.41 [2.16] vs 299.83 [1.94] U/mL; sham, 154.75 [2.02] vs 303.08 [0.35] U/mL; control, 157.08 [1.67] vs 314.50 [1.14] U/mL) compared with saline-treated rats (all, $P < 0.001$). In the test rats, ulcer index was significantly increased and SOD activity was significantly decreased compared with the sham and control groups (both, $P < 0.001$). Pretreatment with vitamin C and melatonin was associated with attenuation of ulcer index and increased SOD activity compared with rats treated with cysteamine alone ($P < 0.001$). There were no significant differences in ulcer index or SOD activity between groups administered vitamin C or melatonin.

CONCLUSIONS: In this experimental study, pretreatment with melatonin or vitamin C in all rats produced significant attenuation of the ulcer index and enhanced SOD activity. Cysteamine-induced duodenal mucosal damage was greater in cholestatic rats compared with sham and control rats. (Curr Ther Res Clin Exp. 2010;71:322–330) © 2010 Elsevier HS Journals, Inc.

KEY WORDS: cysteamine-induced duodenal ulcer, cholestasis, melatonin, superoxide dismutase, vitamin C, rats.

INTRODUCTION

It has been reported that melatonin has a protective effect against indomethacin-induced gastric damage in multiple stress-induced lipid peroxidation.1 Gastric mucosal damage induced by different gastroinvasive agents was found to be significantly greater ($P < 0.001$) in cholestatic bile duct double-ligated rats than in sham rats,2 and the frequency of gastrointestinal ulceration was significantly higher ($P < 0.01$) in the cholestatic population than in the healthy population.3 It has been suggested that gastric mucosa in cholestatic rats is more vulnerable to NSAIDs compared with healthy animals ($P < 0.05$), which may be due to a variety of different factors, including increased free-radical formation.4 Melatonin is a powerful antioxidant and a scavenger of free radicals, such as hydroxyl and peroxyl radicals, that does not undergo redox cycling (repeated reduction and oxidation). Once oxidized, it cannot be reduced to its former state, and therefore, it has been referred to as a “suicidal” antioxidant.5 Melatonin, in different models of in vitro and in vivo oxidative stress, has been found to protect tissues against oxidant damage induced by various free-radical–generating agents.6,7 It has been suggested that melatonin exerts protective and therapeutic effects against cholestatic liver injury and its associated oxidative stress in bile duct-ligated rats through its antioxidant actions as well as its anti-inflammatory effects.8-12 Vitamin C can reduce and thereby neutralize reactive oxygen species (ROS) such as hydrogen peroxide. Vitamin C is an antioxidant agent that undergoes redox cycling that may allow it to act as a pro-oxidant and promote free-radical formation.13 It has a biological role as a reducing agent in a number of hydroxylation reactions and protects essential substances in the body such as proteins, lipids, carbohydrates, and DNA and RNA from damage by free radicals.14 Cysteamine hydrochloride (HCl) has been found to be
the most potent agent for inducing duodenal ulcer, and cysteamine-induced duodenal ulcer in animals is now used to study the antiulcer activity of drugs. The cysteamine used in experimental studies has been found to concentrate in the duodenum. Its ulcerogenic effect may be due to the generation of ROS, the decreasing defense activity of superoxide dismutase (SOD), and increasing duodenal endothelin-1 concentration, which are all associated with decreased duodenal mucosal blood flow. Oxidative stress, enhanced free-radical levels, and an impaired in-cell antioxidant pool are important factors underlying the pathophysiologic mechanisms in a variety of diseases. The superoxide radical and SODs (enzymes that catalyze the dismutation of the superoxide radical anion) have been implicated in many disease states, including inflammatory diseases, diseases of ischemia and reperfusion, neurodegenerative diseases, and cancer.

The present study assessed the protective effects of melatonin and vitamin C against cysteamine-induced duodenal ulcer and their ability to enhance antioxidant enzymatic defense by measuring ulcer index and SOD activity in a cholestatic rat model.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Male adult Wistar rats (n = 72) weighing 200 to 250 g were used in this study. All animals were provided food and water ad libitum and housed 3 per cage at 22 ± 2°C with a controlled 12-hour light–dark cycle. The animals were handled in accordance with the criteria and recommendations of the ethics committee on animal experiments of the Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Animals were divided into 3 groups of 24 animals each: the bile-duct ligated (test) group, the sham group, and the control group. All animals were then further divided into 4 subgroups of 6 animals each that received one of the following: (1) vitamin C (350 mg/kg IP) at 7:30 AM and 11:30 AM plus cysteamine (230 mg/kg SC) at 8:00 AM and 12:00 PM; (2) melatonin (20 mg/kg IP) at 7:30 AM and 11:30 AM plus cysteamine (230 mg/kg SC) at 8:00 AM and 12:00 PM; (3) cysteamine (230 mg/kg SC) at 8:00 AM and 12:00 PM; or (4) saline (1 mL/kg SC) at 8:00 AM and 12:00 PM.

TEST GROUP

Laparotomy was performed under general anesthesia induced by intraperitoneal injection of ketamine (65 mg/kg) and chlorpromazine (20 mg/kg), and the common bile duct was identified, isolated, and doubly ligated. Then, 7 days after surgery, when the group had shown overt jaundice, the animals were divided into the 4 subgroups and treated as described previously. Rats were euthanized via overdose of ether anesthesia 24 hours after the last dose of cysteamine. Blood samples were then collected, duodenums were removed, and ulcer assessment and SOD activity were determined.

SHAM GROUP

Laparotomy was performed under general anesthesia induced by injection of ketamine (65 mg/kg IP) and chlorpromazine (20 mg/kg IP) and the common bile duct was identified, manipulated, and left in situ. The abdominal wall was then closed in
2 layers. Seven days after surgery, the animals were divided into the 4 subgroups and treated as described previously. Rats were euthanized via overdose of ether anesthesia 24 hours after the last dose of cysteamine. Blood samples were then collected, duodenums were removed, and ulcer assessment and SOD activity were determined.

**Control Group**

No surgery was conducted in the control group. The rats were divided into the 4 subgroups and treated as described previously. They were then euthanized via overdose of ether anesthesia 24 hours after the last dose of saline. Blood samples were then collected, duodenums were removed, and ulcer assessment and SOD activity were determined.

**Ulcer Index Measurement and Histopathologic Assessment**

The duodenum (5 cm in length) was removed and the esophagus and duodenum were clamped. Then, after 20 to 30 minutes, the stomach and adjacent duodenum were cut open and washed out with saline. Samples from each group were fixed in 10% neutral buffered formaldehyde for 24 hours. Ulcers were examined by 5× binocular magnifier to assess lesions. Ulcers were scored for intensity using a 4-point scale (0 = no ulcer; 1 = superficial mucosal erosion; 2 = deep ulcer or transmural ulcer; and 3 = perforated or penetrated ulcer). The ulcer index was calculated by using the following formula:

$$U_I = U_N + U_S + U_A \times 0.1,$$

where $U_I$ was ulcer index, $U_N$ was number of ulcers, $U_S$ was ulcer score, and $U_A$ was the ulcer surface area for each duodenum.

For histopathologic assessment, the formalin-fixed specimens were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin, and then histochemical sections were evaluated by light microscopy.

**SOD Activity Assessment**

In all 3 groups, 0.5 mL of blood was drawn from the heart ventricle into a syringe containing EDTA (final EDTA concentration of 1 mg/mL), transferred to plastic tubes, centrifuged for 10 minutes at 3000 rpm, and then aspirated off the plasma. The red blood cells (RBCs) were washed 4 times with 3 mL of normal saline and centrifuged for 10 minutes at 3000 rpm after each wash. Then, 2.0 mL of cold redistilled water was added to the washed, centrifuged RBCs, mixed and left to stand at 4°C for 15 minutes before being subjected to lysis. The lysate was diluted with 0.01 mol/L phosphate buffer (pH = 7.0) and SOD activity was determined using an enzyme kit (RANSOD, Randox Laboratories, Inc., Crumlin, United Kingdom). The method employs xanthine and xanthine oxidase to produce superoxide radicals that react with INT (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride) to form a red formazan dye. The SOD activity was measured photometrically by the degree of inhibition of this reaction at 505 nm and 37°C and expressed as U/mL.
CHEMICALS
The following chemicals were used in the study: melatonin (Albissola Marina, Savona, Italy), vitamin C (Osvah Pharmaceutical Co., Tehran, Iran), chlorpromazine (Tehran-Shimi, Tehran, Iran), ketamine HCl (Pantex, Duizel, the Netherlands), cysteamine HCl (Merck, Darmstadt, Germany), formaldehyde (Merck), SOD kit (Randox Laboratories), and EDTA (Merck).

STATISTICAL ANALYSIS
Data were analyzed statistically by \( t \)-test and expressed as mean (SE). \( P < 0.05 \) was considered statistically significant. Calculations were performed using SPSS version 16 (SPSS Inc., Chicago, Illinois).

RESULTS
TEST GROUP
In animals treated with cysteamine alone in the test group, mean (SE) duodenal ulcer index (4.00 [0.10]) was significantly increased and SOD activity was significantly decreased (146.41 [2.16] U/mL) compared with saline-treated animals (1.17 [0.30] and 299.83 [1.94] U/mL; both, \( P < 0.001 \)) (Table). Rats pretreated with vitamin C or melatonin were associated with attenuation in ulcer index (1.83 [0.16] and 1.67 [0.21], respectively) and increased SOD activity (234.91 [1.65] and 236.66 [1.22] U/mL; \( P < 0.001 \)) compared with rats treated with cysteamine alone. There were no significant differences in ulcer index or SOD activity between the groups pretreated with vitamin C or melatonin.

SHAM GROUP
In rats treated with cysteamine alone in the sham group, mean (SE) ulcer index was 3.83 (0.16) and SOD activity was 154.75 (2.02) U/mL (Table). Pretreatment with vitamin C or melatonin was associated with attenuation in ulcer index (1.67 [0.21] and 1.50 [0.22], respectively) and significantly increased SOD activity (239.50 [1.06] and 242.2 [0.14] U/mL; both, \( P < 0.001 \)). There were no significant differences in ulcer index or SOD activity between the groups pretreated with vitamin C or melatonin.

CONTROL GROUP
In rats treated with cysteamine alone in the control group, mean (SE) ulcer index was significantly higher (3.67 [0.21]) and SOD activity was significantly lower (157.08 [1.67] U/mL) compared with those treated with saline (0 and 314.50 [1.14] U/mL) (Table). Pretreatment with vitamin C or melatonin was associated with attenuation of the ulcer index (1.50 [0.22] and 1.33 [0.21], respectively) and significantly increased SOD activity (242.08 [1.18] and 246.91 [1.83] U/mL; both, \( P < 0.001 \)) compared with rats treated with cysteamine alone. There were no significant differences in ulcer index or SOD activity between the groups pretreated with vitamin C or melatonin.
Table. The effects of vitamin C and melatonin pretreatment on ulcer index and activity of superoxide dismutase (SOD) in rats. Data are mean (SE).

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<tr>
<th>Group</th>
<th>Subgroup</th>
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<tr>
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<td>Vitamin C (350 mg/kg)* + Cysteamine (230 mg/kg)† (n = 6)</td>
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<td>Bile duct ligation (n = 24)</td>
<td>Ulcer index</td>
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<td>SOD, U/mL</td>
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<td>Sham (n = 24)</td>
<td>Ulcer index</td>
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<td>SOD, U/mL</td>
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<td>Control (n = 24)</td>
<td>Ulcer index</td>
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<td>SOD, U/mL</td>
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*Administered at 7:30 AM and 11:30 AM.
†Administered at 8:00 AM and 12:00 PM.
†P < 0.001 versus cysteamine alone.
HISTOLOGIC STUDIES
The microscopic observation of all rats treated with saline showed normal appearance of duodenum mucosa, but rats treated with cysteamine alone showed ulcer with a mucosal defect, which penetrated the muscularis mucosae and muscularis properia. In rats pretreated with vitamin C or melatonin, protection against the histopathologic changes was observed.

DISCUSSION
An adequate balance among the antioxidant enzymes is necessary to minimize the toxic effects of ROS. SOD is one of the main antioxidant enzymes found in aerobic organisms that constitutes a part of the physiologic defense against oxidative stress. It has been suggested that gastric mucosal lesions are associated with a decrease in defense activity against ROS and the protective effect of antioxidants is related to their ability to improve oxidative stress in gastric mucosa. ROS is known to be involved in the pathogenesis of mucosal lesions in the gastrointestinal tract, and free radicals play an important role in these mucosal lesions. The role of ROS has been well established in many disorders. The SOD level is estimated as an index of the antioxidant status. When the generation of ROS overwhelms the antioxidant defense, lipid peroxidation of the cell membrane occurs, which causes disturbances in the cell integrity leading to cell damage. The efforts of the endogenous antioxidant enzymes to remove the continuously generated free radicals initially increase due to an induction, but later, enzyme depletion takes place, resulting in oxidative cell damage. Blood concentration of vitamin C and other antioxidants decreases due to the accelerated consumption in the blood.

In the present study, cysteamine was used as a cytotoxic agent, whose effect partly depends on the generation of hydroxyl radical, and it has been suggested that antioxidants inhibit cysteamine-induced duodenal ulcer. It seems that, in the presence of cysteamine, free-radical production overwhelms antioxidant defense, and this situation causes disturbances in the cell membrane. In the present study, vitamin C and melatonin were used as antioxidants, which inhibited cysteamine-induced duodenal ulcer and increased the activity of SOD in all 3 groups of rats. Histologic analysis confirmed the biochemical results. This effect may be due to increase in enzymatic antioxidant defenses and protection of cell components from ROS-mediated damage.

Limitations of the present study include the open-label design and the small number of rats used in each group. Large and controlled studies in humans are needed to confirm the results.

CONCLUSIONS
Pretreatment with melatonin and vitamin C in all rats produced significant attenuation of the ulcer index and enhanced SOD activity. The results of this experimental study suggest that cysteamine-induced duodenal mucosal damage was greater in cholestatic rats compared with sham and control rats.
ACKNOWLEDGMENTS
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Drs. Rezvanjoo and Rashidi conducted the experimental work. Dr. Jouyban conducted statistical analysis and assisted in the preparation of the manuscript. Dr. Beheshtiha assisted with the SOD activity measurement. Dr. Samini was the study guarantor.

REFERENCES


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