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The effect of thalidomide on ethanol-induced gastric mucosal damage in mice: Involvement of inflammatory cytokines and nitric oxide

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ABSTRACT

Excessive ethanol ingestion causes gastric mucosal damage through the inflammatory and oxidative processes. The present study was aimed to evaluate the protective effect of thalidomide on ethanol-induced gastric mucosal damage in mice. The animals were pretreated with vehicle or thalidomide (30 or 60 mg/kg, orally), and one hour later, the gastric mucosal injury was induced by oral administration of acidified ethanol. The animals were euthanized one hour after ethanol ingestion, and gastric tissues were collected to biochemical analyzes. The gastric mucosal lesions were assessed by macroscopic and histopathological examinations. The results showed that treatment of mice with thalidomide prior to the administration of ethanol dose-dependently reduced the gastric ulcer index. Thalidomide pretreatment significantly reduced the levels of pro-inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6], malondialdehyde (MDA) and myeloperoxidase (MPO) activity. In addition, thalidomide significantly inhibited ethanol-induced nitric oxide (NO) overproduction in gastric tissue. Histological observations showed that ethanol-induced gastric mucosal damage was attenuated by thalidomide pretreatment. It seems that thalidomide as an anti-inflammatory agent may have a protective effect against alcohol-induced mucosal damage by inhibition of neutrophil infiltration and reducing the production of nitric oxide and inflammatory cytokines in gastric tissue.

1. Introduction

Peptic ulcer disease is a common gastrointestinal disorder and is an important health problem in worldwide. The pathophysiology of gastric mucosal injury results from an imbalance between protective factors and aggressive factors. Various damaging agents are involved in the pathogenesis of gastric mucosal injury, including alcohol intake, gastric hydrochloric acid, free oxygen radicals, non-steroidal anti-inflammatory drugs (NSAIDs), stress, and Helicobacter pylori infection [1,2]. It is well known that ethanol causes acute gastric mucosal injuries through different mechanisms, including gastric microcirculatory disturbances, disruption of the gastric mucosal barrier and gastric epithelial cell damage [3,4]. Alcohol can trigger some of the acute inflammatory pathways leading to gastric mucosal injury [5]. It has been shown that neutrophil infiltration into the gastric mucosa has a critical role in the development of gastric mucosal inflammation and damage. Infiltration of neutrophils and mononuclear cells into the gastric mucosa during inflammation stimulates the synthesis and release of several pro-inflammatory mediators. It has been shown that some pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL-10 are involved in the pathogenesis of alcohol mediated mucosal injury [6–9]. NO plays an important role in regulating physiological functions of gastrointestinal tract, however, NO has been implicated as a contributor to tissue injury in the gastrointestinal tract during inflammatory responses. Thus, NO appears to have a dual role in the inflammatory processes [10,11].

A number of compounds with anti-inflammatory activity have been studied to prevent the gastric mucosal injury induced by ethanol in animal models [7–9]. Thalidomide (α-N-phthalimido glutarimide) as an anti-inflammatory agent is effective in the treatment of inflammatory disorders. Thalidomide is a glutamic acid derivative that was first introduced in 1956 as a sedative drug but was withdrawn from clinical use in 1962 due to its teratogenic effects. Thalidomide is now used as a treatment for cutaneous lesions associated with erythema nodosum leprosum (ENL) and multiple myeloma. Thalidomide causes some adverse effects, particularly teratogenicity and peripheral neuropathy. New structural analogues of thalidomide have been developed in order to improve therapeutic efficacy and reducing side effects [12]. Several studies

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have been shown that thalidomide is effective in the treatment of gastrointestinal inflammatory disorders, such as ulcerative colitis and Crohn's disease. These studies indicate that thalidomide can suppress the production of TNF-α, IL-1β, IL-6 and NO in gastrointestinal tissue [13–16]. In addition, our previous studies have also confirmed that thalidomide exerts its anti-inflammatory effects by suppressing the production of proinflammatory cytokines [17–19]. TNF-α has a major role in the pathophysiology of ethanol-induced gastric mucosal inflammation and damage [7,9,20]. It has been previously reported that inhibition of TNF-α significantly reduced the severity of gastric damage in cirrhotic rats [21]. In the present study, we further investigated the effects of thalidomide on the production of pro-inflammatory cytokines and NO, and histopathological changes in a mouse model of ethanol-induced gastric mucosal injury.

2. Materials and methods

2.1. Animals

Male and female albino mice, weighing 26–30 g, were kept in our animal house under controlled conditions. The animals were allowed free access to tap water and standard laboratory food ad libitum. Mice were acclimated to the procedure room for at least one week before the experiments were started. Mice were fasted overnight, but allowed free access to water, before induction of gastric mucosal ulcers.

2.2. Materials

Thalidomide (>98%), tetramethylbenzidine, Tris–HCl buffer, dimethyl sulfoxide, hydrogen peroxide, thiobarbituric acid, trichloroacetic acid and 1,1,3,3-tetraethoxypropane were purchased from Sigma–Aldrich. Ethanol (97%) was purchased from Taghtir Co. (Iran). Protease inhibitors (Complete Mini tablets) were purchased from Roche (Germany). The enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α, IL-1β, IL-6 were purchased from eBioscience. The colorimetric assay kit for nitric oxide (NO) was obtained from Enzo Life Sciences.

2.3. Ethanol/HCl-induced gastric mucosal damage

Oral administration of ethanol/HCl has long been used as an experimental method to induce gastric mucosal injury in animals. HCl accelerates the progress of ulcerogenesis and enhances gastric injury caused by ethanol [22,23]. Animals were randomly divided into five experimental groups with 8–10 mice in each group as follows: the normal control group received vehicle orally; the ethanol control group received a single dose of acidified ethanol; the thalidomide + ethanol group received 30 or 60 mg/kg thalidomide orally and acidified ethanol; and the thalidomide group received thalidomide alone. Thalidomide was dissolved in 0.5% carboxyl methylcellulose (CMC) and administered orally 1 h before application of the ulcerogenic agent. One hour after oral treatment with thalidomide the animals received acidified ethanol (60% ethanol/0.3 M HCl, 0.1 ml/10 g body weight) by the oral route to induce gastric mucosal ulcer. The animals were euthanized 1 h after administration of ethanol/HCl and the stomachs were removed and opened along the greater curvature. The stomachs were rinsed with cold saline to remove the gastric contents and blood clots in order to evaluate the extent of gastric mucosal damage. After that, the gastric tissue samples were frozen in liquid nitrogen and stored at −80 °C for later biochemical analysis.

2.4. Determination of macroscopic gastric mucosal damage

Macroscopic scoring of gastric tissue samples was performed under light microscopy. The degree of gastric mucosal damage was expressed as an ulcer index and calculated according to the quantity and the diameter of the erosion [22]. Ulcer index = Σ(A) + (2B) + (3C) (A is the number of small erosions up to 1 mm; B is the number of erosions up to 3 mm; C is the number of linear erosions greater than 3 mm). The mean scores for each experimental group were calculated and then compared statistically.

2.5. Histopathological procedure and assessment

For pathological assessment, a small specimen of each stomach was fixed in 10% formalin solution, and then embedded in paraffin. Embedded sections were cut using the microtome at a thickness of 4 μm and stained with hematoxylin and eosin (H&E). The histopathological changes were evaluated by an experienced pathologist according to the method previously reported [24]: (1) mucosal edema (score 0–4), (2) hemorrhage (score 0–4), (3) inflammatory cell infiltration (score 0–3), and (4) epithelial cell loss (score 0–3).

2.6. Determination of cytokines

The gastric tissue samples were homogenized in ice-cold Tris–HCl buffer (pH = 7.4) containing protease inhibitors. The homogenized samples were centrifuged at 20,000×*g* for 20 min in a refrigerated centrifuge at 4 °C. The supernatants were collected and frozen at −80 °C until assay. The cytokine levels of TNF-α, IL-1β and IL-6 in tissue samples were determined using ELISA kits according to the manufacturer's instructions. Cytokine levels in the samples were expressed as pg cytokine/mg protein.

2.7. Determination of myeloperoxidase (MPO) activity

The activity of MPO in gastric tissue samples was determined by measuring the H_2O_2 – dependent oxidation of tetramethylbenzidine (TMB). In its oxidized form, TMB is blue, which was measured spectrophotometrically at 655 nm. The homogenates were sonicated and centrifuged for 20 min at 20,000×*g*. The supernatants were collected and used for the enzyme activity assay. The reaction mixture for analysis consisted of 50 μL sample, 50 μL TMB solution (15 mM, dissolved in DMSO), and 100 μL of hydrogen peroxide (25 mM) diluted in phosphate buffer (50 mM, pH 5.4). The assay was performed in a 96-well microtiter plate. The reaction was started by adding hydrogen peroxide and the rate of change in absorbance was measured at 655 nm using a microplate reader. One unit of MPO activity was defined as the amount of enzyme that decomposed 1 μmol of H_2O_2 per min at 25 °C. The results were expressed as units of MPO/mg protein.

2.8. Determination of tissue NO levels

An aliquot of the supernatant was used to determine nitric oxide (NO) levels. The gastric tissue content of nitrate as a marker of total NO concentration were measured by a colorimetric assay kit based on Griess reaction, according to the manufacturer's instructions. Results were expressed as nmol/ mg protein.

2.9. Determination of malondialdehyde (MDA) levels

The gastric tissue content of MDA was measured according to the thiobarbituric acid (TBA) method [19]. Briefly, 100 μl of the supernatant was added to a reaction mixture containing 100 μl of 20% (w/v) trichloroacetic acid (TCA) and 200 μl of TBA (0.05 M). The reaction mixture was incubated in a boiling water