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Apocynin protects against ethanol-induced gastric ulcer in rats by attenuating the upregulation of NADPH oxidases 1 and 4

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ABSTRACT

Gastric ulcer is a common gastrointestinal disorder affecting many people all over the world. Absolute ethanol (5 ml/kg) was used to induce gastric ulceration in rats. Apocynin (50 mg/kg) was given orally one hour before the administration of absolute ethanol. Omeprazole (20 mg/kg) was used as a standard. Interestingly, apocynin pre-treatment provided 93.5% gastroprotection against ethanol-induced ulceration. Biochemically, gastric mucin content was significantly increased with apocynin pre-treatment. This finding was further supported by alcian blue staining of stomach sections obtained from the different treated groups. Also, gastric juice volume and acidity were significantly reduced. Apocynin significantly ameliorated ethanol-induced oxidative stress by replenishing reduced glutathione and superoxide dismutase levels as well as reducing elevated malondialdehyde levels in gastric tissues. Besides, ethanol-induced pro-inflammatory response was significantly decreased by apocynin pre-treatment via reducing elevated levels of pro-inflammatory markers; interleukin-1β, tumor necrosis factor-α, cyclooxygenase-2 and inducible nitric oxide synthase. Additionally, caspase-3 tissue level was significantly reduced in apocynin pre-treated group. Interestingly, NADPH oxidase-1 (NOX-1) and NOX-4 up-regulation was shown to be partially involved in the pathogenesis of ethanol-induced gastric ulceration and was significantly reversed by apocynin pre-treatment. Gastroprotective properties of apocynin were confirmed by histopathological examination. It is worth mentioning that apocynin was superior in all aspects except gastric mucin content parameter where it was significantly increased by 13.5 folds in the omeprazole pre-treated group. This study was the first to show that apocynin is a promising gastroprotective agent against ethanol-induced gastric ulceration, partially via its anti-oxidant, anti-inflammatory, anti-apoptotic effects as well as down-regulating NOX-1 and NOX-4 expression.

1. Introduction

Gastric ulcer is a major multifactorial gastrointestinal disorder. Indeed, the high consumption of alcohol and non-steroidal anti-inflammatory drugs (NSAIDs) greatly contribute to the formation of gastric ulcer [1]. The therapeutic strategies of gastric ulcer include improvement of gastroprotective mechanisms and/or reducing stress factors and gastric juice production [2]. However, most of the drugs used are not completely effective in healing gastric ulcers [3]. Accordingly, deep understanding of gastric ulcer pathogenesis and searching for more effective gastroprotective agents is very important. The pathogenesis underlying gastric ulcer is a very complicated process. Gastric ulcer develops due to breaks in gastric mucosal barrier [1]. Indeed, the balance between acid secretion and factors responsible for mucosal defense is disrupted [2]. Besides, oxidative stress and inflammation are greatly involved in ethanol-induced gastric ulcer pathogenesis. In agreement with this, agents possessing anti-oxidants and anti-inflammatory activities had greatly showed promising gastroprotective effects [4–6]. Moreover, ethanol was shown to induce apoptotic death of the underlying gastric mucosa cells which is also linked to oxidative stress [4,6]. NADPH oxidases (NOXs) are involved in reactive oxygen species generation; particularly superoxide anion [7]. There is evidence that up-regulation of NOXs is involved in gastric ulcer induced by aspirin [8] and diethyldithiocarbamate [9], where sources of superoxide anion were shown to play a major role. Moreover, NOX polymorphism was shown to greatly determine the risk for gastroduodenal diseases in Japan [10].

Apocynin is a phytochemical which is structurally related to vanillin. It was found that apocynin is a powerful NOX inhibitor and consequently, has an anti-oxidant activity that was expressed in a

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variety of disease animal models such as hepatitis [11], lung fibrosis [12] and myochardial ischemia [13]. Besides, Mouzaoui et al. [14] showed that apocynin pre-treatment conferred protection against TNFα-induced acute colitis in mice. Additionally, apocynin inhibited NOX production that was shown to be greatly involved in the pathogenesis of both acute and chronic colon inflammation induced by dextran sulfate sodium *in vivo* [15] and *in vitro* [16]. Also, apocynin was found to possess an anti-cancer activity in a variety of cancer cell lines [17,18]. Furthermore, Hart et al. [19] showed that apocynin administration prevents the formation of tubercle bacteria-induced ulcerative skin lesions. However, apocynin gastroprotective role has never been investigated.

Thus, the aim of this study was to assess the possible gastroprotective effects of apocynin against ethanol-induced gastric ulceration in rats. Besides, the underlying gastroprotective mechanisms were explored. Furthermore, the study was directed to elucidate whether NOX-1 and NOX-4 are possibly involved in the pathogenesis of gastric injury induced by ethanol.

2. Material and methods

2.1. Chemicals

Apocynin, omeprazole and absolute ethanol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). D-galactose, D-mannose, orcinol were obtained from Al-Dawleah Chemical Co. (Cairo, Egypt). Concentrated sulfuric acid was purchased from Al-Gomhorea Chemical Co. (Cairo, Egypt). All chemicals and solvents were of highest grade commercially available.

2.2. Animals

The study was conducted according to the ethical guidelines (Ain Shams University, Egypt). Male Wister rats (200–220 g) were obtained from the Nile Company for Pharmaceutical and Chemical industries, Egypt. The rats were housed in an air-conditioned atmosphere, at a temperature of 25 °C with alternatively 12 h light and dark cycles. The animals were acclimated two weeks before experimentation and were kept on a standard diet and water, *ad libitum*. Standard diet pellets (El-Nasr Chemical Company, Abu-Zaabal, Egypt) contained not less than 20% protein, 3.5% fat, 6.5% ash and a vitamin mixture. All the animal experiments were approved by the ethical committee of the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

2.3. Experimental design

The overnight fasting animals were divided randomly into four groups (n = 6). Group 1 served as normal control and was given vehicle only (8% Tween 80 in water). Group 2 was given absolute ethanol (5 ml/kg) orally and served as the positive ulcer control. Group 3 was pre-treated orally with apocynin at a dose of 50 mg/kg one hour before giving absolute ethanol. Group 4 was given 20 mg/kg omeprazole one hour before administration of absolute ethanol. One hour after ethanol administration, the animals were sacrificed. The gastric contents were collected and centrifuged at 5000 rpm for 10 min. Gastric juices obtained from the different treated groups were used to measure gastric juice volume, titrable acidity and mucin content as well. Stomach tissues were dissected, opened along the greater curvature and stretched on paraffin bed for macroscopic inspection. Stomach homogenates were used to assess oxidative stress markers (reduced glutathione, lipid peroxides and superoxide dismutase), pro-inflammatory markers; interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Also, caspase-3 tissue levels were determined in the homogenates obtained from the different groups. Besides, the study was concerned with the estimation of tissue contents of NOX-1 and NOX-4. In addition, stomach samples were taken from the different experimental groups for histopathological examination as well as histochemical staining with alcian blue stain.

2.4. Assessment of gastric mucosal injury

Ethanol-induced gastric ulceration appeared as elongated bands of hemorrhagic lesions [20–22]. The areas of ulcerated lesions were determined using image J analysis software (Image J, 1.46a, NIH, USA) and the percentages of the ulcerated area relative to the total stomach area were calculated. The percentage of gastroprotection was determined according to the following formula:

$$\text{Percentage of gastroprotection}(\%) = \frac{\text{Ulcerated area(positive ulcer control)} - \text{Ulcerated area(treated)}}{\text{Ulcerated area(positive ulcer control)}} \times 100$$

2.5. Assessment of gastric juice acidity

Acid concentration (mEq/l) in gastric juice samples was determined by being titrated against 0.01 N sodium hydroxide solution, using phenolphthalein as an indicator, till the first color changes from transparent to pink [20,23].

2.6. Assessment of mucin content

The gastric mucin content was determined in gastric juice samples taken from the different treated groups [20,24] and was expressed in terms of mg/ml hexoses. 250 μl of diluted gastric juice sample was mixed with equal volume of 1.6% orcinol solution and 2 ml 60% sulfuric acid. Samples were then placed in boiling water bath for 10 min, allowed to cool and the developed color was measured spectrophotometrically at 425 nm.

2.7. Assessment of oxidative stress in gastric tissue

Levels of reduce glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) were assessed in the stomach homogenates taken from the different treated groups. GSH was measured using GSH assay kit (Biodiagnostic, Egypt) [25]. MDA assay kit (Biodiagnostic, Egypt) was used to assess lipid peroxidation [26]. The activity of SOD in tissue homogenates were assessed using SOD assay kit (Biodiagnostic, Egypt) according to the manufacturer's instructions.

2.8. Determination of protein content in gastric tissue

The protein content in stomach homogenates was determined using bovine serum albumin as a standard according to the method