PRE-ADMINISTRATION OF CURCUMIN PREVENTS HYPERHOMOCYSTEINEMIA IN ETHANOL-INDUCED GASTRIC ULCER

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ABSTRACT

Curcumin is a prototype natural product that has been widely recognized as an antioxidant and anti-inflammatory agent. In this study, we aimed to evaluate the potential role of curcumin supplementation in attenuating the aggressive effect of alcohol in experimental induced gastric ulcer. Forty male albino rats weighing 150-180 g were classified into four groups including control, curcumin, ethanol and treated groups. Oxidant and antioxidant parameters were estimated, liver and kidney function were measured, tumor necrosis factor - α (TNF-α) was determined by ELISA and homocysteine was estimated by HPLC using reversed phase column and UV detector that was performed at 260 nm. Stomach malondialdehyde (MDA) and nitric oxide (NO) levels were significantly increased by ethanol consumption compared to control group beside the reduction of reduced glutathione (GSH) and the elevation of homocysteine and TNF-α. Whereas, pre-treatment of curcumin prevented the hyperhomocysteinemia and the elevation of TNF- α resulting in a depletion of gastric ulcer. We concluded that, curcumin is considered a promising supplement in attenuating gastric ulcer through its important role in depletion of oxidative stress and inflammation.

KEYWORDS: gastric ulcer, curcumin, hyperhomocysteinemia, HPLC, ethanol.
INTRODUCTION
Gastric ulcer, abrasion in the lining of the stomach or duodenum occurring when the mucosal epithelium exposed to acid and pepsin.\(^1\) Factors that could increase the incidence of gastric ulcer in the global population include stress, smoking, Helicobacter pylori, non-steroidal anti-inflammatory drugs (NSAIDs) and high consumption of alcohol.\(^2\)

The previous studies suggested that, gastric ulcer is the result of a disruption in the normal balance between aggressive factors such as gastric acid, pepsin, and bile salts and defensive aspects [prostaglandin (PG), mucosal blood flow, mucus, and bicarbonate]\(^3\); whereas, oxidative stress and reactive oxygen species (ROS) are important factors that can cause intracellular damage. It was clearly observed that, ulcers are commonly associated with lipid peroxidation and oxidative damage in the mucosa because ROS may cause oxidative damage of biological macromolecules such as protein, lipids and DNA.\(^4\)

Experimentally, gastric ulcer could be induced by chemical agents like NSAIDs\(^5\), aspirin and alcohols. Several studies demonstrated that, ethanol consumption elevates homocysteine\(^6\), the toxic agent that induces oxidative stress and subsequent injury in indothelial cells\(^7\); in addition to elevation of the free fatty acids that enhance the enzymes which catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes.\(^8\)

Curcumin is a prototype natural product that has been widely recognized as an antioxidant and anti-inflammatory agent.\(^9\) The polar structure and low molecular weight of curcumin allow it to effectively cross the blood–barrier. In obese and diabetic rodents, curcumin can also improve insulin sensitivity, increase the antioxidant status of pancreatic β-cells, and ameliorate dyslipidemia\(^10,11\), which are associated with a reduction in plasma free fatty acids (FFA) levels.

In this study, we aimed to evaluate the potential role of curcumin supplementation in attenuating the aggressive effect of alcohol induced experimental gastric ulcer.

MATERIALS AND METHODS
Materials
Chemicals
Homocysteine standard (HPLC grade) and curcumin were purchased from Sigma-Aldrich Company, St. Louis, MO, USA.
All other chemicals were HPLC grade.

**Experimental animals**

Forty male albino rats weighing 150-180 g were obtained from the animal house of National Research Centre, Giza, Egypt, and fed a standard commercial diet (control diet) purchased from the Egyptian Company of Oils and Soaps. Water was freely available ad-libitum.

**METHODS**

The animal experimental protocol was approved by the Ethical Committee of National Research Centre, Egypt. After the acclimatization period, rats were randomly assigned into four groups; each group was comprised of ten rats as follows:

**Group I:** healthy rats received a vehicle and served as a control group.

**Group II:** healthy rats received curcumin (dissolved in 0.9% NaCl) at a dose of 80 mg/kg body weight / day orally for 2 weeks and served as a curcumin group.

**Group III:** healthy rats received a vehicle for 2 weeks before receiving ethanol and served as ethanol group.

**Group IV:** healthy rats received curcumin (dissolved in 0.9% NaCl) at a dose of 80 mg/kg body weight / day orally for 2 weeks before receiving ethanol and served as treated group.\(^{12}\)

To induce gastric ulcer, rats were fasted for 12 hours and received 1 ml absolute ethanol / rat once to induce gastric ulcer as modified from the method described previously\(^{13}\); two hours later, blood was withdrawn from the retro-orbital venous plexus of the eye from all groups using capillary tubes and collected in dry clean tubes.

Stomach was removed quickly; gastric mucosal injury was assessed by volume of gastric secretion (in ml) and pH of gastric secretion. The stomach was then stored for subsequent biochemical procedures.

**Preparation of tissue homogenate**

One gram from frozen tissue was cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na\(_2\)HPO\(_4\) and 0.7 g of NaH\(_2\)PO\(_4\) dissolved in 500 ml deionized water (pH 7.4) , centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was removed and used in biochemical parameters estimation.\(^{14}\)
Biochemical assays
Serum liver enzymes alanine amino-transferase (ALT) and aspartate amino-transferase (AST) were measured colorimetrically according to the previous method\cite{15}, also, blood urea and serum creatinine were measured.\cite{16, 17}

Determination of stomach MDA
Lipid peroxidation products in the stomach homogenates was assayed by measuring the level of malondialdehyde using the method described by Ruiz-Larrea et al.\cite{18} where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a complex characterized by a red-color and measured at \(532\) nm.

Determination of reduced glutathione
Reduced glutathione (GSH) was determined in stomach homogenates spectrophotometrically according to the method described previously.\cite{19}

Determination of nitric oxide
Nitric oxide measured as nitrite was determined using Griess reagent, according to the previous method.\cite{20}

Determination of serum homocysteine
Homocysteine was estimated by high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (G131A model).

Sample extraction
Briefly, 400 μl from serum samples were treated with 30 μl of 1.2 mol/L trichloroacetic acid (TCA), mixed well and incubated in ice for 30 min to precipitate protein. After centrifugation for 20 min at 4000 rpm and 4°C, supernatants were filtered through hydrophilic 0.45 μm polyvinyliden fluoride (PVDF) membrane filter.

HPLC condition
50 μl from the filtered supernatant were injected into HPLC; separation was achieved on reversed phase column (C18, 25, 0.46 cm i.d. 5 μm). The mobile phase consisted of 40 mmol/L sodium phosphate monobasic monohydrate; 8 mmol/L heptanesulfonic acid and 18% (v/v) methanol pH was adjusted to 3.1 by addition of phosphoric acid ; filtered two times through a 0.45-μm membrane filter. The mobile phase was then delivered at a flow rate of 1 ml/min at 40°C. UV detection was performed at 260 nm. Serial dilutions of standards were
injected into HPLC, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. Concentrations in samples were obtained from the standard curve.\(^{[21]}\)

**RESULTS AND DISCUSSION**

The aim of this study was to investigate and clarify the role of curcumin, a polyphenolic non-enzymatic antioxidant agent, against gastric ulcer. The goal was to establish an experimental model of gastric ulcer induced by ethanol consumption.

Ethanol consumption in our work significantly increased gastric secretion volume and acidity, in addition to the elevation of serum ALT, AST, urea and creatinine compared to control group (table 1). These effects of alcohol consumption on liver and kidney functions might have been partly a consequence of the intestinal damage.

All of these damaging factors promote oxidative stress by increasing the formation of ROS and inducing the depletion of oxidative defenses in the cells. MDA represents an end product of the peroxidation of polyunsaturated fatty acids and related esters within cell membranes and it is currently regarded as a reliable index of oxidative tissue damage.\(^{[22]}\)

**Table 1: Levels of gastric secretion volume, gastric secretion PH, Liver functions and kidney functions in different studied groups.**

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Gastric secretion PH</th>
<th>Gastric secretion volume</th>
<th>ALT U/L</th>
<th>AST U/L</th>
<th>Blood urea m mol/L</th>
<th>Serum creatinin mg/ dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>6.7±0.14</td>
<td>0.3±0.05</td>
<td>16.0±2.08</td>
<td>20.0±5.1</td>
<td>7.8±0.18</td>
<td>0.52±0.03</td>
</tr>
<tr>
<td>Curcumin group</td>
<td>6.9±0.20(^b)</td>
<td>0.2±0.03(^b)</td>
<td>13.6±1.6(^b)</td>
<td>31.3±2.6(^b)</td>
<td>7.9±0.47(^b)</td>
<td>0.55±0.01(^b)</td>
</tr>
<tr>
<td>Ethanol group</td>
<td>3.3±0.44(^a)</td>
<td>5.1±0.44(^a)</td>
<td>58.3±5.2(^a)</td>
<td>90.6±15.6(^a)</td>
<td>22.5±0.43(^a)</td>
<td>2.1±0.3(^a)</td>
</tr>
<tr>
<td>Treated group</td>
<td>5.3±0.29(^a,b)</td>
<td>3.0±0.28(^a,b)</td>
<td>28.3±4.1(^a,b)</td>
<td>52.6±3.4(^a,b)</td>
<td>15.1±2.3(^a,b)</td>
<td>1.0±0.32(^a)</td>
</tr>
</tbody>
</table>

Data presented as mean ± SE

Significant p value < 0.05

a : significant difference compared to control group

b : significant difference compared to ethanol group

n : number of cases = 10
Ethanol has an important role in gastrointestinal diseases because it induces gastric mucosal injury by disrupting the barrier function of the mucus, the main protective barrier against pepsin and gastric acid.\(^{[23]}\) Ethanol can also induce direct oxidative damage in gastric mucosal tissues by increasing hydroxyl radical production and lipid peroxidation in the gastric mucosa\(^{[24]}\) as was found in the current study, thus, stomach MDA and NO levels were significantly increased by ethanol consumption compared to control group (Fig.1,2) beside the reduction of reduced glutathione (GSH) (Fig.3).

![Fig.1: Stomach malondialdehyde levels in different studied groups](image1)

![Fig.2: Stomach nitric oxide levels in different studied groups](image2)
In this study, proinflammatory markers (TNF-α and homocysteine) were significantly increased by ethanol consumption compared to the control group (fig. 4, 5).
Ethanol administration impairs methionine metabolism by inhibiting the enzyme methionine synthase (MS); this inhibition results in impaired remethylation of homocysteine to produce methionine and, in turn, decreases the production of S-adenosylmethionine (SAM) and accumulation of homocysteine in the liver and circulation. The thiol group of homocysteine is oxidized in plasma resulting in generation of reactive oxygen species. Indeed, homocysteine has the ability to inhibit antioxidant enzymes expression\cite{25} and increase in oxidative stress as was found in our work. Thus, there was a positive correlation was found between homocysteine and oxidative stress parameters (MDA and NO) as well as a negative correlation between homocysteine and GSH (Fig.6)
Fig. 6: Positive correlation between A) homocysteine and MDA and B) homocysteine and NO, whereas, a negative correlation is observed between C) homocysteine and GSH.

A previous study indicated that homocysteine is a potent inducer of duodenal ulcers, and its cytotoxicity partly depends on the generation of H$_2$O$_2$ and ROS. This process increases the duodenal mucosal flow and causes tissue ischemia and hypoxia.\cite{26}

TNF-α, an adipocytokine, is known to stimulate chronic lipolysis in primary or differentiated adipocytes.\cite{27} The elevation of TNF-α may be associated with increased plasma FFA levels. The treatment of adipocytes with curcumin did not affect basal lipolysis but rather blocked the lipolytic action mediated by TNF-α.\cite{28}

The antiulcer activity of curcumin in ethanol-induced gastric ulcer model is shown in Figures 7. Results showed that, rats pretreated with curcumin before being given ethanol had significantly reduced areas of gastric ulcer formation compared to ulcer control group. Ethanol consumption produced extensive visible black hemorrhagic lesions of gastric mucosa.
Our results showed that curcumin at a dose of 80 mg/kg inhibited ethanol-induced gastric injury in animals and maintained the biochemical parameters of gastric mucosal tissue, such as GSH which is an endogenous antioxidant, and its properties are related to the presence of the thiol group in its structure. Glutathione reacts with peroxides and toxic oxygen radicals to protect cells from damage. The quantification of GSH levels in the gastric tissue of treated rats shows that, the levels of reduced glutathione were increased in the presence of curcumin. This result clearly demonstrates that curcumin protected the gastric mucosa against damage caused by ethanol.

It was found that, GSH plays an important role in gastric mucosal defence through many actions, including the maintenance of mucosal blood flow.\[29\]

Indeed, curcumin supplementation effectively attenuated MDA and NO elevation in treated group compared to ethanol group ( fig.1 ,2 ) that may be due to the fact that, curcumin, a polyphenolic non-flavonon compound is pharmacologically active and has antioxidant anti-inflammatory and anti-proliferative activities, thus, the antioxidant activity of curcumin is equivalent to vitamins C and E.\[30\]

Several mechanisms were postulated for curcumin to play as anti-inflammatory and antioxidant agent. These mechanisms may be related to its effect in the reduction of free fatty acids (FFA) in circulation.

Curcumin may decrease the plasma free fatty acids (FFA) concentration through increased fatty acid beta-oxidation activity\[31\], decreased endogenous polyunsaturated fatty acid synthesis\[32\], reduced macrophage infiltration in white adipose tissue\[33\], inhibited TNF- \(\alpha\)
expression\textsuperscript{[34]} and reduced hyperhomocysteinemia. These various biological activities of curcumin, taken together, contribute to its effect of lowering the plasma FFA and hence decreasing the enzymes which catalyze the biosynthesis of thromboxanes, prostaglandins and leukotrienes\textsuperscript{[8]} resulting in a reduction of stomach injury.

CONCLUSION
From our results, we concluded that, curcumin is considered a promising supplement in attenuating gastric ulcer through its important role in depletion of oxidative stress as well as inflammatory action.

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