INTRODUCTION

Gastric ulcer is a painful lesion that is caused by erosion of the mucosal layer of stomach or necrosis of the surface of a tissue due to sloughing of inflammatory necrotic tissue [1]. In normal situations, stomach maintains a balance between protective factors and aggressive factors [2]. When the balance between aggressive factors (such as acid and pepsin) and defense mechanisms (such as mucus, bicarbonate, blood flow and mucosal turnover) is shifted in favor of aggressive factors, peptic ulcers are manifested [3]. Factors, such as stress, smoking, alcohol usage, nutritional deficiencies, and frequent ingestion of non-steroidal anti-inflammatory drugs (NSAIDs), have been shown to be associated with an increased incidence of gastric ulcer [4]. Several mechanisms to explain the pathophysiology of gastric ulceration development associated with frequent NSAID use have been suggested, including inhibition of prostaglandin synthesis [5] and increased mucosal production of inflammatory cytokines (e.g. tumor necrosis factor alpha [TNF-α]) [6]. TNF-α is a well-characterized stimulus for adhesion molecule expression [7]. Appleyard et al. [8] suggested that TNF-α might be the key signal for NSAID-induced neutrophil adherence within the gastric microcirculation. Other possible mechanisms of NSAIDs-induced gastropathy include overproduction of reactive oxygen species and lipid peroxidation [9]. In addition, it was reported that the increased ulcerogenic response to indomethacin (IND) is mediated through the reduction of constitutive nitric oxide synthase (cNOS) activity [10]. Overall, it is obvious that many factors are involved in the pathogenesis of IND-induced gastric ulcers such as leukocyte infiltration, free radicals formation, and disturbance in NO production in gastric tissues. Therefore, any compound isolated from natural sources and exhibits antioxidant properties and has the capacity to inhibit neutrophil infiltration as well as modify NO level might be of good value in the protection against IND-induced gastric ulcer.

Genistein (GEN), a soy-derived isoflavone, has received a huge attention as it has been demonstrated to be effective in the prevention of today’s most prevalent chronic diseases, namely cardiovascular diseases, osteoporosis, and hormone-related cancers [11]. GEN is widely available in the Asian diet and is believed to be a potent antioxidant [12]. Scientists believe that isoflavones, by their antioxidant nature, are capable of neutralizing free radicals and reducing the inflammatory reactions [12]. In addition, GEN has been demonstrated to activate peroxisome proliferator-activated receptors PPARs [13], a subgroup of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are critical in regulating insulin sensitivity, adipogenesis, lipid metabolism, inflammation, and blood pressure. Furthermore, in double-blinded, randomized, clinical trials, GEN was proven to increase plasma NO breakdown products, decrease endothelin-1 levels, and enhance endothelial-dependent vasodilation [14]. It also reduced homocysteine and C-reactive protein levels [15]. GEN by its multi-mechanisms is documented to be effective in the prevention of cardiovascular and renal diseases; however, its role in NSAID-induced peptic ulcer is not tested yet.

The aim of this study was to investigate the anti-ulcerative effect of GEN against IND-induced gastric ulcer and whether it is linked to oxidant/antioxidant/anti-inflammatory effects. Accordingly, ulcer index, myeloperoxidase (MPO), and NO were measured in gastric tissues.

In addition, oxidative stress biomarkers such as reduced glutathione (GSH), lipid peroxidation (malondialdehyde [MDA]) levels, superoxide
Four groups of animals (n=6) were used. Animals were divided into 4 groups, each of 8 rats:

Group 1: Rats in this group (control) were given the vehicles orally by intragastric gavage.

Group 2: Rats in this group were intragastrically treated with GEN (10mg/kg) [16].

Group 3: Rats in this group were treated orally by an intragastric tube with 48 mg/kg of IND dissolved in 5% NaHCO₃. This dose of IND is within the range reported in the literature to elicit macroscopically visible acute hemorrhagic erosions and ulcers in the stomach of normal rats [17].

Group 4: Rats in this group were 1 week pretreated with GEN orally [16] before IND at the same previous doses.

After 6 hrs of IND treatment, the rats were sacrificed by decapitation; their stomachs were rapidly removed and processed for evaluation of gastric mucosal injury.

Animals subjected to pylorus ligation
To examine the effects of IND and or GEN on gastric secretion, studies were performed in different groups of pylorus ligated rats as described by Rajashkhekar et al. [18]. Four groups of animals (n=6) were used in this experiment. Under short anesthesia with diethyl ether, the abdomen was opened by a midline laparotomy and the duodenum was exteriorized. The pylorus was ligated, the abdominal incision was closed with clips, and the animals were allowed to recover from anesthesia. The first control group was given the vehicle orally. The second group was treated orally with GEN (10 mg/kg). The third group was given IND 48 mg/kg orally; the fourth group was pretreated orally with GEN for 1 week before IND. The animals were sacrificed at 6 hrs following IND administration. The contents of stomachs were emptied; the collected volume was measured, centrifuged, and analyzed for acidity using rat enzyme-linked immunosorbent assay kit (rat TNF-α ELISA kit, Biosource, Belgium) according to the manufacturer’s protocol. TNF-α concentrations were expressed as pg/g tissue.

Determination of gastric mucosal MPO concentration
MPO concentration was determined in rat gastric tissue homogenate using rat enzyme-linked immunosorbent assay kit (rat MPO ELISA kit, Wkea Med Supplies Corp, China) according to manufacturer protocol. MPO concentrations were expressed as ng/g tissue.

Estimation of oxidative stress biomarkers in gastric mucosal tissues
GSH content of gastric tissues was determined in the homogenates using commercially available GSH assay kit. Lipid peroxidation was calculated by estimating the level of MDA. MDA was determined in rat gastric homogenates using commercially available MDA assay kit. The results were expressed as nmol MDA/g tissue. The enzymatic activity of SOD was assessed in gastric tissue homogenate using commercially available SOD assay kit. SOD activity was determined by calculating the difference between auto-oxidation of pyrogallol alone and in the presence of SOD enzyme. GSH, MDA, and SOD assay kits were obtained from Biodiagnostic, Dokki, Giza, Egypt.

Histopathological evaluation
The stomach of each animal was removed, fixed in 10% formalin for 24 hrs and processed for histopathological examination. Four-micrometer-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination using conventional protocol [20]. A minimum of 8 fields for each stomach section were examined and assigned for the severity of changes by an observer blinded to the treatments of the animals.

Statistical analysis
Data were expressed as the means±SE. One-way analysis of variance (ANOVA) test followed by Tukey’s post-hoc test was used to determine the statistically significant difference between multiple comparisons. A probability value of p<0.05 was considered to indicate statistical significance.

RESULTS
Gastric ulcer index and gastric juice acidity
The gastric ulcer index is calculated as the sum of the lengths in millimeters of all hemorrhagic and ulcerative lesions. The ulcer index (mm) measured in the gastric mucosa of the IND-treated group was (45±2.45). Administration of GEN markedly reduced the ulcer index to (15±1.42) resulting in 66.67% reduction in gastric lesions induced by IND in rat stomachs (p<0.001) (Fig 1a). Oral administration of GEN alone to rats did not show hemorrhagic or ulcerative lesions (Fig 1a). In terms of gastric juice acidity, IND increased gastric mucosal acidity (mEq/l) to give a value of (101±9.3) compared to control value of (35±16.6). Pre-treatment of rats with GEN showed no significant effect in gastric acidity compared to IND group (Fig 1b). Oral administration of GEN alone also did not affect gastric acidity (Fig 1b).
Quantitative estimation of gastric mucosal contents of TNF-α, MPO, and NO expressed as total nitrite/nitrate.

Oral administration of IND significantly increased the pro-inflammatory mediator TNF-α by more than 3 folds as compared with the control group (Fig. 2a). Pre-administration of GEN before IND markedly reduced the elevated TNF-α contents by 50% (p<0.001) (Fig. 2a). Rats treated with IND orally showed an increase in MPO contents to give a value of (40±1.3) compared to the control group (12±0.93) indicating enhancement of neutrophils infiltration into mucosal tissues. Administration of GEN one week before IND reduced the elevated MPO level to (23±0.94) (p<0.001) (Fig. 2b). In addition, NO level expressed as total nitrite/nitrate (nmol/g tissue) was lowered by about 46% to give a value of (350±22.2) compared to control (651±21) after IND administration. Pre-treatment with GEN significantly elevated the decreased total nitrite/nitrate levels in gastric mucosa to (577±22) (p<0.001) (Fig. 2c).

Determination of oxidative stress biomarkers (GSH, MDA, and SOD) in gastric mucosal tissues

IND in this study produced a decrease in GSH levels (nmol/g tissue) from 23±1.5 to reach 12±1. GEN prevented the IND-induced decline in reduced GSH content and restored its normal level to 20±1.6 (p<0.05) (Fig. 3a). Moreover, IND caused an elevation in lipid peroxide (MDA) levels (nmol/g tissue) in gastric tissues from 1.5±0.1 to 8.5±0.46. GEN was able to normalize the elevated MDA levels to 2±0.16 (p<0.001) (Fig. 3b). SOD activity (U/mg protein) was decreased in gastric tissues of IND-treated rats from 15.0±1.3 to 8.5±0.46. However, the reduced SOD activity was increased to 12±1.1 following GEN administration (p<0.05) (Fig. 3c). Administration of GEN (alone) did not show any significant effects on GSH, MDA contents or SOD activity, respectively (Fig. 3a-c).

Assessment of gastric mucosal injury morphologically and histologically

No visible hemorrhagic and/or ulcerative lesions were seen in the control and GEN groups (Fig. 4a and c). Sections from control and GEN groups showed the normal histological structure of the gastric mucosa glandular and non-glandular layers (Fig. 4b and d). Oral administration of IND for 6 hrs induced a gross gastric mucosal damage in rats. These hemorrhagic and ulcerative lesions were seen as red streaks (Fig. 4e). Histologically, this group showed erosion associated with diffuse leukocyte cells infiltration that was observed in lamina propria of the mucosal layer. This was accompanied by hypertrophy in the muscularis of the glandular portion (Fig. 4f). Pre-treatment with GEN markedly reduced the visible hemorrhagic lesions induced by IND in rats’ stomach (Fig. 4g). Histologically, pre-treatment with GEN before IND maintained the normal histological structure of glandular portion (Fig. 4h) with mild edema in the junction between glandular and non-glandular portions (Fig. 4h). Most of the histopathological lesions induced by IND and ameliorated by GEN in gastric tissues were summarized in Table 1.
Data were obtained from investigation of four histological sections from the gastric tissues of the control group (CO), GEN-treated group, IND-treated group, and their combination (GEN+IND). The severity of changes using scores of 0 (absent) and + (mild level): <25% of the total fields examined revealed histopathological alterations. ++ (moderate level): <50% of the total fields examined revealed histopathological alterations. +++ (severe level): <75% of the total fields examined revealed histopathological alterations.

### Table 1: The severity of histopathological alterations in gastric tissues of experimental groups of rats treated with GEN, IND and their combination (GEN+IND) in comparison to the control group (CO)

<table>
<thead>
<tr>
<th>Groups/histopathological alterations</th>
<th>CO</th>
<th>GEN</th>
<th>IND</th>
<th>GEN+IND</th>
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<tbody>
<tr>
<td>Congestion</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Inflammatory cells infiltrations</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Muscular hypertrophy</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Edema</td>
<td>0</td>
<td>0</td>
<td>+++</td>
<td>+</td>
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</table>

GEN: Genistein, IND: Indomethacin, +: Mild level, ++: Moderate level, +++: Severe level

DISCUSSION

NSAIDs are valuable agents in the treatment of arthritis and other musculoskeletal disorders, and as analgesics in a wide variety of clinical scenarios. Unfortunately, their use has been limited by their association with mucosal injury to the upper gastrointestinal tract, including the development of peptic ulcer disease along with its complications, most notably upper gastrointestinal hemorrhage, and perforation [21]. IND is an NSAID that was reported to induce gastric ulcer through multi-mechanisms [22].
Our results showed that IND produced gastric ulcer in rats as indicated from gastric ulcer index. According to Sabiu et al. [22], the abnormal elevation of gastric juice acidity by IND may also participate in the augmentation of the severity of gastric ulcer. Choi et al. [23] reported that inflammatory mediators, such as TNF-α, are involved in the pathogenesis of IND-induced gastric ulcer and is one of the aggressive factors in ulcerogenesis. It was documented that TNF-α level is elevated in gastric tissues of IND-treated rats [23], and this came online with our findings. Furthermore, it was proved that there is a relation between TNF-α and PGE2. It was found that exogenous administration of PGE2 produced an anti-oker effect by preventing the IND-induced TNF-α increase [24].

There is evidence that TNF-α stimulates neutrophil infiltration into the gastric mucosa, and its overproduction increases the risk of gastric ulcer and cancer [24]. MPO (a neutrophil infiltration index) level is augmented following IND administration suggesting a role of TNF-α in neutrophil cells infiltration and in the development of gastric ulcer [25]. On the other hand, NO produced by eNOS or NOs plays a cytoprotective role against any injuries insults that harm the gastric tissues possibly through a reduction of leukocyte adhesion and maintenance of gastric blood flow and any reduction in its content will expose the tissues to the damaging effects of IND [25]. Our results are matched with the aforementioned findings as IND administration to rats has led to an increase in MPO levels and a reduction in NO levels.

Another mechanism that is involved in the pathogenesis of gastric ulcer is oxidative stress mechanism. Based on certain studies, IND causes an overproduction of ROS [25,26]. This was similar to the findings of this study in which the administration of IND to rats reduced the GSH levels and SOD activity in gastric tissues. This was also consistent with what was reported by Kaplan et al. [27]. On the other hand, IND elevated the MDA level in this study; MDA is accepted as a marker of lipid oxidation [25].

GEN, a class of phytoestrogens, known as isoflavones, is mostly found in legumes. It has attracted attention because of its beneficial effects on the prevention of metabolic disorders related to cardiovascular disease (CVD), obesity, cancer, and diabetes [28,29]. GEN has been extensively established as a multifunctional agent through enhancing the antioxidant defense system and anti-inflammation response [30]. Obviously, GEN in this study has showed a protective effect against IND-induced ulcers in rats. Pre-treatment with GEN significantly reduced the gastric ulcer index (mm) that was elevated after IND treatment. Gastric ulcer index is the measure of hemorrhagic and ulcerative lesions that are produced by IND in gastric tissues. Pro-inflammatory mediators such as TNF-α are involved in the pathogenesis of gastric ulcers. GEN was reported to decline the elevated TNF-α in d-galactosamine induced fulminant hepatic failure in Wistar rats [31], an effect that came in agreement with our study as the administration of GEN markedly reduced the elevated TNF-α levels in gastric tissues. The infiltration of neutrophils (measured as MPO index) into ischemic myocardium exacerbates myocardial damage on reperfusion [32], whereas drugs such as GEN that inhibit neutrophils activity or function [33] may reduce the inflammatory damaging effect that seen after neutrophils infiltration. In our study, pre-treatment with GEN reduced the MPO activity, giving an indication that GEN is a neutrophils activity inhibitor. Therefore, GEN may inhibit infiltration of neutrophils into gastric tissues, which produce and secrete TNF-α leading to a reduction in the pro-inflammatory mediators (TNF-α) release into gastric tissues. NO produced in gastric tissues has a cytoprotective role. GEN pre-treatment enhanced NO levels that were reduced by IND in gastric tissues. Wang et al. [34] reported that GEN augments NO levels in rats subjected to four-vessel global cerebral ischemia through enhancement of eNOS phosphorylation/activation, supporting our results and emphasizing the role of NO in gastric protection against IND gastric lesion.

Oxidative stress mechanism is also involved in the pathogenesis of gastric ulcer induced by IND. GEN is reported to have an antioxidant mechanism [35]. GEN was found to increase GSH levels and SOD activity and reduced the elevated MDA levels in mouse liver [36]. Our results are matched with the aforementioned findings, GEN increased GSH levels, SOD activity and reduced the elevated MDA contents in gastric tissues that were deteriorated after IND treatment.

Based on the above findings, GEN ameliorated the IND-induced gastric ulcers through multi-mechanisms as indicated from the improvement of biochemical analysis. Our results are further supported by the histopathological analysis that reveals alleviation of pathological lesions (e.g., erosion, diffuse leukocyte cells infiltration, and hypertrophy in the muscularis of the glandular portion) after treatment with GEN. In contrast to what is expected, GEN has no effect on the gastric acidity that was elevated by IND, indicating that GEN has a gastro-protective effect without effect on gastric acidity.

CONCLUSION

These results indicate that GEN has a protective effect against IND-induced gastropathy probably through inhibiting inflammatory mediators, neutrophil infiltration, suppression of oxidative stress generation and replenishing of NO levels regardless of gastric acidity.

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REFERENCES


