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GASTROPROTECTIVE EFFECT OF STANDARDIZED GINKGO BILOBA EXTRACT (EGB761) AGAINST INDOMETHACIN-INDUCED GASTRIC ULCERS IN RATS

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Abstract

The study aims to investigate the anti-ulcer activity of a natural product; standardized Ginkgo biloba extract (EGb761) against gastric ulcer induced by the non-steroidal anti-inflammatory drug (NSAID); indomethacin and its possible antioxidant and anti-inflammatory effect when it is administered alone or in combination with the standard drug misoprostol. Single oral dose of misoprostol, EGb761 and their combination, were administerted 30 min. before indomethacin-induced acute gastric ulceration. Four hours later, animals were sacrificed, their stomach were tested for the presence of ulcers, then the following markers were assessed: gastric levels of malondialdehyde (MDA), prostaglandin E2 (PGE2) and superoxide dismutase (SOD) activity, in addition to plasma catalase activity, and serum CRP level. Indomethacin produced a high incidence of gastric ulcerations. All pretreatments significantly reduced ulcer index, the highest was the combination, then misoprostol, followed by EGb761 EGb761 has powerful ulcer healing properties. These effects may be due to its possible antioxidant and anti-inflammatory actions. Simultaneous administration of EGb761 with misoprostol improved their ulcer healing effects, however, an mechanistic interaction occurred between the two drugs. Therefore, another mechanism may be responsible for the ulcer healing properties of the combination.

Keywords
EGb761; NSAID; indomethacin; misoprostol; ulcer index; PGE2; CRP; catalase; SOD

1.Introduction

Gastric Ulcer (GU) is a very common gastrointestinal disease which may lead to dangerous complications and even death. It is accounting for an estimated 15 mortality out of every 15,000 complications yearly in the world 2. GU affects approximately 10% of the population worldwide. Gastric ulcers associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) remain a major clinical problem (1) and (2).

The continuous generation of prostaglandins by cyclooxygenase isoenzymes in the gastric mucosa helps to maintain an adequate mucosal blood flow and also stimulates the generation of mucus (3). NSAIDs inhibit cyclooxygenase and thereby reduce the intrinsic ability of the mucosa to resist injury induced by endogenous and exogenous aggressors. They can induce gastric ulcers through various processes, including generation of reactive oxygen species, initiation of lipid peroxidation, infiltration of leukocytes, induction of apoptosis, and inhibition of prostaglandin synthesis (4). Decreased prostaglandin level impairs almost all aspects of gastroprotection and increases acid secretions which in turn, aggravate the ulcer (5).

The dominant factor in the treatment of ulcers induced by anti-inflammatory drugs is the mucosal resistance against oxygen-derived free radicals, which play an important role in the pathogenesis of acute experimental gastric lesions (6). Thus, much attention has been recently focused on ROS contents, such as superoxide, hydroxyl radicals (OH) and singlet oxygen (7). Therefore, antioxidant defense systems, including antioxidant enzymes, foods and drugs are important in the prevention of the toxic ROS effects (8).

Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers. Thus, there is currently a resurgence of interest in the use of natural bioactive products by the general public, with many healthy subjects and patients taking them for the prevention and treatment of multiple conditions, including gastrointestinal disorders. Unfortunately, current evidence of the scientific validity of many of these traditional and commercial compounds is severely limited (9).

Ginkgo biloba, a member of the family Ginkgoaceae, is used medically today as a standardized preparation GbE (EGb 761) which contains 240 mg/g flavonoids (ginkgo-flavone glycosides) and 60 mg/g terpenoids (ginkgolides and bilobalides). The flavonoids act as free radical scavengers, especially for oxygen-derived free radicals, such as OH, O_2-, RO, and ROO, and to neutralize ferryl ion-induced peroxidation. The terpenoids is known as an antagonist of platelet-activating factor, which implicates in the processes of platelet aggregation and arterial thrombosis, acute inflammation, allergic reactions and cardiovascular insufficiency. GbE is well-known as a strong free radical scavenger (10) and (11).

Misoprostol (cytotec), a synthetic prostaglandin E_1 analogue that was designed for the prevention and treatment of peptic ulcer associated with the use of

nonsteroidal anti-inflammatory drugs (12). The most commonly reported adverse effect of long term taking misoprostol to reduce the risk of NSAID-induced gastric ulcers is diarrhea and abdominal pain (5). It showed a rapid increase in healing ulcers through enhancement of endogenous PGE_2 and elevation in COX-2 expression, so we used it as a reference anti-ulcer drug (13).

Therefore, the aim of the present work is to study the gastroprotective activity of EGb761 and its combinations with the anti-ulcer drug misoprostol, this will give an insight about the role of antioxidant and anti-inflammatory activities in gastroprotective effect of this natural product.

2. Materials and methods

2.1. Drugs and chemicals

Gum acacia powder, carboxymethyl cellulose (CMC), liometacen (Nile Co., Cairo, Egypt), cytotec (Pfizer, USA), Standardized Ginkgo biloba extract (EGb761) (Mepaco, Cairo, Egypt) and heparin (Nile Co., Cairo, Egypt).

Antioxidants assay kits (Biodiagnostic, Egypt).Also, immunoassay kits: PGE2 ELISA kit (Neogen Co., Lexington KY, USA) and hs-CRP Accubind ELISA kit (Monobind Inc., Lake Forest, CA, USA) following the instructions of the manufacturer. The chemicals used were all of analytical reagent grade. All drugs and reagents were prepared immediately before use.

2.2. Preparation of the reference drug

A specific dry weight of misoprostol was macerated with a mortar and pestle in double distilled water containing gum acacia 2% (w/w) to provide the drug (14).

2.3. Animals

Adult male rats weighing about (180–220 gm) were raised in the animal house of the Faculty of Pharmacy, Zagazig University. The animal room was well ventilated with a 12 h light/dark cycle throughout the experimental period. They were maintained in clean, sterile, polypropylene cages and fed with regular rat chow and water ad libitum and were left to accommodate for one week. All rats were deprived of food for 18 h before the expirement, but were allowed free access to water. In the day of the experiment; their weights had been measured. The study was approved by the institutional ethical committee, which follows the guidelines of CPSCEA (Committee for the Purpose of Control and Supervision of Experimental on Animals), which complies with international norms of INSA.

2.4. Experimental protocol for indomethacin-induced ulcers

Fourty rats were randomly classified into five groups (eight rats per each);

(i) Control group; receiving the vehicle orally 1% CMC–water solution.
(ii) Indomethacin group ; in which acute gastric ulcers were induced by administration of indo (62.5 mg /kg, p.o) in water vehicle (15).
(iii) Misoprostol + indo group; in which rats were pretreated with misoprostol (100µg/kg, p.o.) suspended in 2% gum acacia.

(iv) EGb761 + indo group; in which rats were pretreated with Ginkgo biloba extract (500mg/kg, p.o) suspended in 1% CMC–water solution
(v) EGb761 + Misoprostol + indo group; a combination group receiving the same doses as above.

All pretreatments are administered orally 30 min. prior to induction of gastric ulcers by indomethacin. Four hours later, rats were anaesthetized with diethylether (16). Blood is collected in heparinized and non heparinized tubes, centrifuged and the supernatants were obtained for biochemical analysis. The animals were sacrificed by cervical dislocation, the stomachs were removed, and opened along the greater curvature and then washed with saline. Gastric mucosa was examined for ulcers with the help of magnifying lens and expressed as ulcer index, then stomach lesions immediately frozen in liquid nitrogen and stored at −70 °C until determination of different parameters.

2.5. Determination of ulcer index (UI)

After taking digital pictures of the mucosal surface of each stomach for macroscopical examination, The ulcers were scored according to the method of (17) and assessed on the basis of their dimensions: Deep circular ulcers more than 8 mm = 10 ; 7–8 mm = 8 ; 6–7 mm = 7 ; 5–6 mm = 6 ; 4–5 mm = 5 ; 3–4 mm = 4 ; 2–3 mm = 3 ; 1–2 mm = 2 and 0–1 mm = 1. The deep linear ulcers more than 10 mm in length = 6 and linear ulcer less than 10 mm in length = 3. The score for each single lesion were then summed up for the determination of ulcer index. We calculated the protective ratio (%) according to the following formula (10) and (18):

Preventive ratio (%) = (a-b)/a and multiplied by 100
a: the ulcer index of the ulcerated group
b: the ulcer index of the experimental group

Protective effects of EGb761 and its combinations with misoprostol were compared with the results obtained from the indomethacin and misoprostol groups.

2.6. Biochemical analysis

After the measurement of gastric lesions, glandular segments of stomach were removed and a 10% homogenate was prepared and subjected to biochemical analysis.

2.6.1. Lipid peroxidation biomarker: gastric malondialdehyde (MDA) level

Malondialdehyde (MDA), a marker for lipid peroxidation was estimated in gastric tissues by the thiobarbituric acid (TBA) method (19), and results are expressed as nanomol MDA per gram wet tissue (nmol g tissue^{-1}).

2.6.2. Antioxidant biomarkers: plasma catalase activity and gastric superoxide dismutase (SOD) activity

The catalase activity was estimated in plasma by measuring the rate of decomposition of H_2O_2 at 240 nm (20), and results are expressed as micromole per litre of plasma (μmol L^{-1}).

The superoxide dismutase (SOD) activity was estimated in gastric tissues by the riboflavin photoreduction method (21),

and results are expressed as unit per gram wt tissue (U g tissue^{-1}).

2.6.3. Inflammatory biomarkers: gastric Prostaglandin E_2 (PGE_2) level and serum C-reactive protein (CRP) level

Prostaglandin E_2 (PGE_2) level was estimated in gastric tissues by enzyme-linked immunosorbent assay (ELISA), and results are expressed as nanogram per gram wet tissue (ng g tissue^{-1})

C-reactive protein (CRP) level was estimated in serum by enzyme-linked immunosorbent assay (ELISA), and results are expressed as microgram per milliliter of serum (µg ml^{-1})

2.7. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) for eight rats per experimental group. Statistical analysis was performed with SPSS statistical software program the version 16.0. One-way analysis of variance (ANOVA) followed by post hoc test (LSD) was used to compare the mean values of quantitative variables among the groups. The p value less than or equal to 0.05 were considered statistically significant.

3. Results

Our macroscopical examination of the stomach mucosa showed that acute gastric ulcers induced by indomethacin (indo, 62.5mg/kg, p.o.) in male rats. All pretreatments; misoprostol (100µg/kg), standardized Ginkgo biloba extract (EGb 761, 500mg/kg) and their combination significantly reduced the ulcer index, in comparison with the indomethacin group.

The combination group demonstrated the highest significant inhibition of gastric ulcers (٨٥,٤٥ %), followed by misoprostol (٧٩,٦٩ %), then EGb761 (٧٤,٥٤ %), as shown in **Fig 1, Fig 2** and **Table 1**

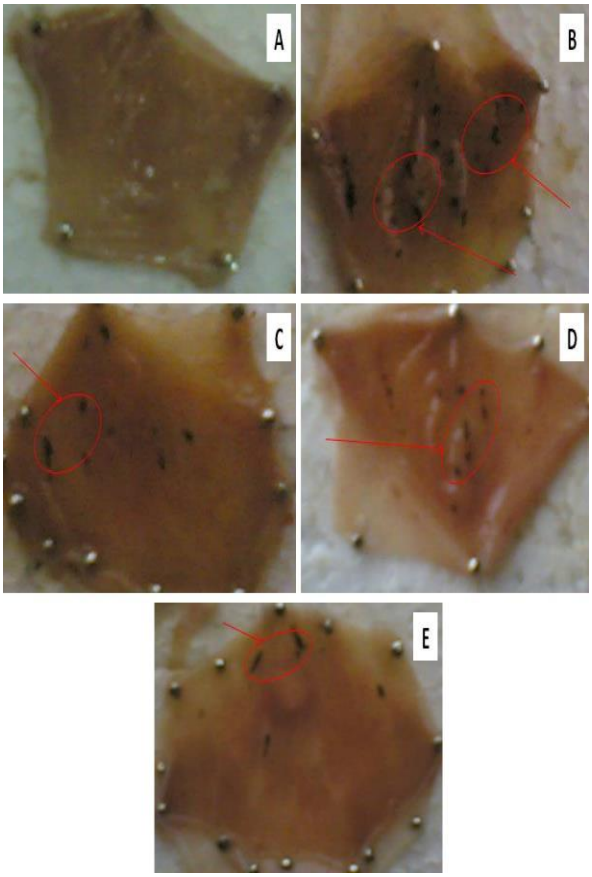

Fig. 1. Pictures of rat stomachs cut along the greater curvature obtained from:
A: control group ; **B**: indomethacin group ; **C**: misoprostol + indo group ; **D**: EGb 761 + indo group ; **E**: EGb 761 + misoprostol + indo group

The combination group demonstrated the highest significant inhibition of gastric ulcers, followed by misoprostol, then EGb761

Treatments	Ulcer index (mm)	Preventive (%)
Control	٠	100
Indomethacin	٥,٠٤ ٥٥* ±	-
Misoprostol + indo	١١,١٧ a ± ٣,٧٩	٧٩,٦٩
EGb 761 + indo	١٤ a ٥,٧٨ ±	٧٤,٥٤
EGb 761 +Misoprostol + indo	٨ a ٥,١١ ±	٨٥,٤٥

Table 1. Anti-ulcerogenic effect of single oral pretreatments of

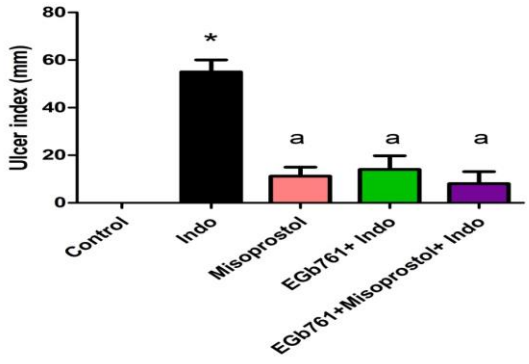

Fig 2. The anti-ulcerogenic effect of single oral pretreatments of misoprostol (100µg/kg), EGb 761 (500mg/kg) and their combination against indomethacin-induced gastric ulcers in rats

misoprostol (100µg/kg), EGb 761 (500mg/kg) and their combination against indomethacin-induced gastric ulcers in rats

Values are presented as mean ± S.E
***** Significantly different from control group at $p \leq 0.05$
a Significantly different from indomethacin group at $p \leq 0.05$

Values are presented as mean ± S.E
***** Significantly different from control group at $p \leq 0.05$
a Significantly different from indomethacin group at $p \leq 0.05$

Results of the biochemical analysis are shown in Fig 3, Fig 4 and Fig 5

Indomethacin group produced a significant increase in gastric MDA levels compared to the control group. In addition, indomethacin caused a severe oxidative stress with a significant increase in serum catalase activity and a significant decrease in gastric SOD activity. Moreover, Indomethacin caused severe inflammation accompanied by a significant decrease in gastric PGE2 level and a significant increase in plasma catalase activity, compared to the control group

All pretreatments had no significant effect on gastric MDA level, compared to indomethacin group, as shown in Fig 3

Pretreatment with misoprostol had a remarkable antioxidant effect as misoprotol produced a significant increase in gastric SOD activity and a significant decrease in plasma catalase activity. In addition, misoprostol had an anti-inflammatory effect through producing a significant increase in gastric PGE2 level, and a moderate decrease in

serum CRP level, compared to indomethacin, as shown in Fig 4 and Fig 5

Pretreatment with EGb761 had an antioxidant effect through producing a significant decrease in plasma catalase activity without affecting gastric SOD activity. In addition, EGb761 had an anti-inflammatory effect, through producing a significant increase in gastric PGE2 level, and a significant decrease in serum CRP level, compared to indomethacin group, as shown in Fig 4 and Fig 5

Pretreatment with the combination had an antioxidant effect through producing a significant decrease in plasma catalase activity without affecting gastric SOD activity. However, the combination had a mild anti-inflammatory effect through producing a slight decrease in serum CRP level and had no effect on gastric PGE2 level, compared to indomethacin group, as shown in Fig 4 and Fig 5

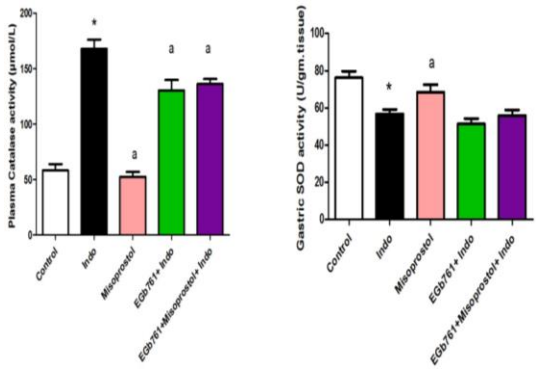

Fig 3. Effect of single oral pretreatments with: misoprostol (100µg/kg), EGb761, (500mg/kg) and their combination on gastric MDA level

Values are presented as mean ± S.E

***** Significantly different from control group at $p \leq 0.05$
a Significantly different from indomethacin group at $p \leq 0.05$

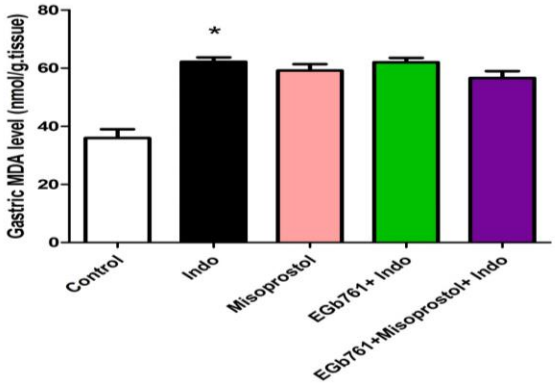

Fig 4. Effect of single oral pretreatments with: misoprostol (100µg/kg), EGb761, (500mg/kg) and their combination on the antioxidant markers: gastric SOD activity, and plasma catalase activity

Values are presented as mean ± S.E
***** Significantly different from control group at $p \leq 0.05$
a Significantly different from indomethacin group at $p \leq 0.05$

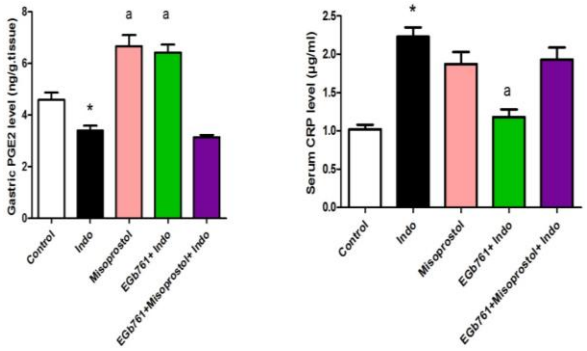

Fig 5. Effect of single oral pretreatments with: misoprostol (100µg/kg), EGb761, (500mg/kg) and their combination on the inflammatory

markers gastric PGE2 levels and serum CRP level

Values are presented as mean ± S.E
***** Significantly different from control group at $p \leq 0.05$
a Significantly different from indomethacin group at $p \leq 0.05$

4. Discussion and conclusion

The effects of combinations of drugs on human health have recently become important issues because many patients take more than one drug at the same time. Naturally, drugs that reduce the side effects of NSAIDs should be selected for patients taking NSAIDs who require treatment with other drugs (22).

The present work demonstrated that indomethacin administration induced a severe mucosal ulceration associated with significant increase in MDA level which has no influence in our results as all pretreatments didn't show any significant change in its level, probably owing to the use of single dose and shortage of time.

Catalase is very effective in high-level oxidative stress and protects cells from hydrogen peroxide produced within the cell. The enzyme is especially important in the case of limited glutathione content or reduced GPx activity (23). According to the present results, the plasma levels of catalase was found to be increased in indomethacin group as compared with control group. This increase may be due to an increase in plasma H_2O_2 and OH^- level, occurred by inhibition of peroxidases (24). The activity of this enzyme was significantly decreased by all pretreatments, which can be attributed to the decrease in plasma H_2O_2 and OH levels. In view of the present results, it can be concluded that all pretreatments possess a reducing effects against the oxidative damage with no additive antioxidant effect in the combined groups.

SOD is considered as the first line of defense against oxygen toxicity and the central regulators of ROS levels by catalyzing the decomposition of superoxide, the first but most abundant ROS, into hydrogen peroxide and water (23). Numerous studies have demonstrated that GbE exerts potent antioxidant activity by acting as scavenger of free radicals, e.g., superoxide anions, hydroxyl radicals and nitric oxide, to protect antioxidant defence system (25, 26 and 27). However, in our experiment gastric SOD activity wasn't affected by EGb761 and its combination with misoprostol groups, while misoprostol produced a significant increase in gastric SOD activity. It is possible that EGb761 antioxidant effect is linked to another mechanism and interacted with the misoprostol induced rise in gastric SOD activity when combined together, thus no additive antioxidant effect was noticed.

Misoprostol and EGb761 had a significant increase on gastric PGE2 level. Interestingly, their combination had no effect on gastric PGE2 level. It is possible that the two drugs interacted and eliminated each other's anti-inflammatory action on PGE2.

C-reactive protein (CRP) is an acute-phase reactant that originates from the liver. CRP has many clinical and

biological effects and can be used for the diagnosis and follow-up of different inflammatory and traumatic processes (28). It is a marker of inflammation. All pretreatments decreased serum CRP level, but this decrease is significant only with EGb761, indicating that combination had no additive anti-inflammatory effect.

In conclusion, this study provides evidence that these drug formulations afford gastroprotection against indomethacin induced ulcers through their antioxidant and anti-inflammatory properties. EGb761 or misoprostol had powerful ulcer healing, antioxidant and anti-inflammatory effects. However, their combination has only improved their ulcer healing effects, leading us to conclude that concomitant administration of misoprostol with EGb761 possessed the highest anti-ulcer activity, possibly through another mechanism, which needs further investigation. Still, it is recommended to separate between the time of administration of EGb761 and misoprostol

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