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(Original Article)

Empagliflozin protects against indomethacin plus pyloric ligation-induced peptic ulcer in rats

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ABSTRACT

Peptic ulcer (PU) is a common gastrointestinal disorder that causes damage to the stomach and duodenal mucosal lining. The goal of this research is to look into the impact of empagliflozin (Empa) on peptic ulcers utilizing an indomethacin (Indo) non-diabetic rat model. Adult male Wistar rats were used and divided into three groups. Group I (control group), group II (Indo group) rats received Indo at dose 100 mg/kg/day (p.o) to induce PU, group III (Empa + Indo group) rats received Empa at dose 10 mg/kg (p.o.) daily for 14 days and received 100 mg/kg/day (p.o) of Indo 1 hr before scarification. Before indomethacin was given to any of the animals, pyloric ligation was performed in order to collect gastric juice. PU was assessed by histopathology and inflammatory mediators were measured using the ELISA technique including COX-2, PGE2, pepsin, TNF-α, and NF-κβ. Indo-treated rats had a marked decrease in COX-2 and PGE2. Moreover, Indo-induced PU was inferred from cytoskeletal changes and was attributable to the overexpression of TNF-α, NF-κB, and pepsin. Empa reversed these effects.

Conclusion: Empa can alleviate PU induced by Indo through the reduction of COX-2, pepsin, TNF-α, and NF-κβ and elevating gastroprotective PGE2.

Keywords: Empagliflozin; peptic ulcer; COX-2; PGE2, inflammation.

1. Introduction

PU is a prevalent condition affecting the gastrointestinal system of humans, characterized by injuries to the mucosal lining of the stomach and duodenum. The onset of PU occurs due to an imbalance in the physiological equilibrium between harmful and protective factors within the gastrointestinal tract (1). The causative factors of this disorder encompass Helicobacter pylori, frequent utilization of non-steroidal anti-inflammatory drugs (NSAIDs), tobacco smoking, ethanol ingestion, malnutrition, and psychological stress (2). It has been shown that NSAIDs are responsible for 25% of incidences of stomach ulcers (1, 3).

NSAIDs are often used to alleviate symptoms including pain, fever, and inflammation. There are several potential adverse reactions to these medications, including gastrointestinal issues such as stomach mucosal erosions, ulcerations, bleeding, and perforations. The risk of bleeding increases with the severity of preexisting peptic ulcers (4). However, it is well-known that NSAIDs exert effects because they block cyclooxygenase (COX), hence reducing the formation of prostaglandin (PG) (5). Reduced PG levels are associated with the etiology of gastroduodenal mucosal ulcers (6), which is the primary complication of low PG levels. Several changes also take place in the mucosa including a reduction in mucus secretion, bicarbonate secretion is inhibited, mucosal blood flow is reduced, microvascular structures are altered, microvascular damage occurs (7), neutrophils infiltrate the area and increase in acid and pepsinogen production occur (8). One of the primary causes of mucosal lesions caused by NSAIDs is gastrointestinal damage, which is largely mediated by reactive oxygen species, particularly superoxide radical anions and hydroxyl radicals (9).

Compared to other frequently used NSAIDs, indomethacin (Indo) (**Figure 1**) possesses a greater propensity to cause gastric harm (10). Due to its potent analgesic properties, this medication is commonly prescribed as a non-selective NSAID for the treatment of migraines and various inflammatory conditions (2). The efficacy of (Indo) has been constrained by its adverse effects, including ulceration and damage to the gastric mucosa (11). These adverse reactions are linked to the impairment of the gastric mucosal barrier, provocation of oxidative stress, stimulation of lipid peroxidation, inhibition of mucus secretion, induction of programmed cell death, infiltration of white blood cells, and stimulation of inflammatory processes (12).

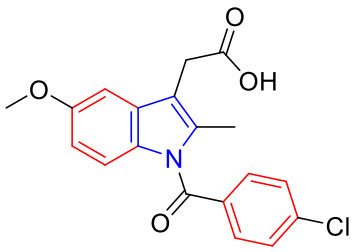

Figure (1): Chemical structure of indomethacin (Indo)

Clinical usage of the SGLT2 inhibitor, empagliflozin (Empa) (**Figure 2**), has been shown to be effective in the treatment of type 2 diabetes mellitus (13). Empa reduces inflammation in the heart, liver, and kidneys, according to many studies in animal models of insulin resistance and diabetes mellitus (14, 15). One of the major advantages of Empa for those with type 2 diabetes is a reduction in mortality (16). Recent data shows that the therapeutic advantages of Empa may occur independently of diabetes effects since we have shown that it improves cardiac outcomes in non-diabetic models of heart failure by lowering myocardial inflammatory activation (17). Therefore, empagliflozin's anti-inflammatory properties suggest it may mitigate the development of indomethacin-induced peptic ulcers in non-diabetic rats. Based on this, this research aims to look into the impact of empagliflozin on peptic ulcers utilizing an indomethacin rat model.

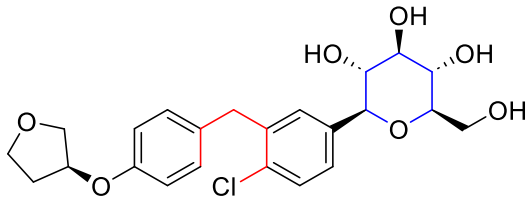

Figure (2): Chemical structure of empagliflozin (Empa)

2. Experimental

2.1. Materials

Empagliflozin (Empa) was obtained from Hikma Pharma®, Cairo, Egypt. Indomethacin (Indo) was obtained from El Nile Pharmaceutical Company, Cairo, Egypt.

2.2. Animals

Male Wistar rats (150–200 g body weight) were kept in a controlled environment (22°C ± 2°C and constant humidity) under a 12-hour light/dark cycle. A standard diet and water were freely available.

2.3. Ethics approval statement

The research methods adhered to the National Institutes of Health's standards for laboratory animal care and use (18). The present animal experiment followed the ARRIVE standards (19).

2.4. Experimental design

Rats were randomly divided into three groups; each contained six animals. All animals were treated for 14 consecutive days (**Figure 3**). The treatment and groups were: the ***control group***, administered isotonic saline (1 ml/kg/day; p.o) for 14 days; ***Indo-induced PU group***, administered a single oral gavage of indomethacin (Indo) (100 mg/kg/day; p.o) 1hr before scarification (10); the ***Empa + Indo group*** (Empa) (10 mg/kg/day; p.o) for 14 days before administration of a single oral gavage of (Indo) (100 mg/kg/day; p.o) 1hr before scarification (10, 20).

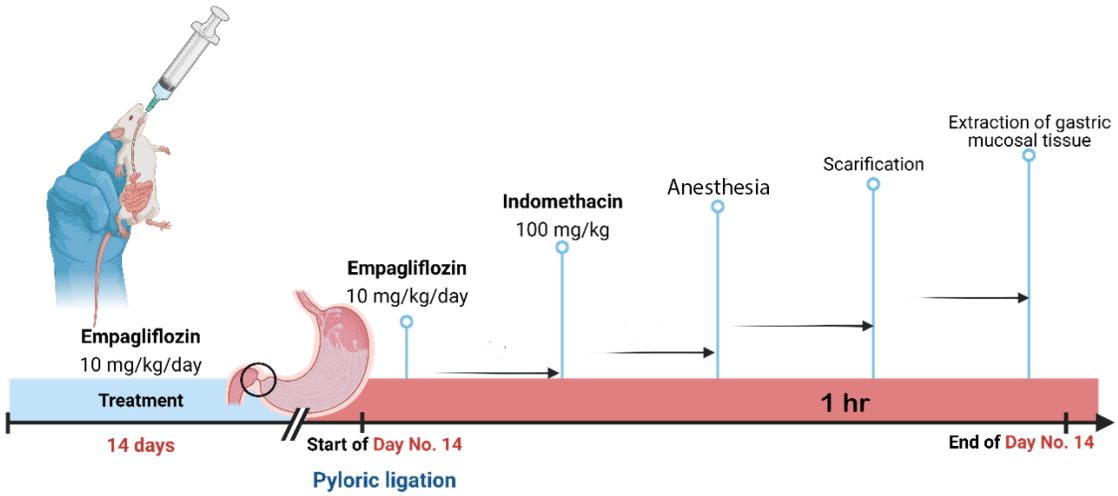

Figure (3): Schematic diagram showing the experimental timeline

At the end of the experiment, the rats were weighed and then anesthetized by thiopental sodium (30 mg/kg; i.p) (Egyptian International Pharmaceutical Industries Company [EIPICO] ®, Tenth of Ramadan City, Egypt.) (21). The rats were then sacrificed by decapitating, and gastric mucosal tissues were quickly dissected (**Figure 3**). Tissues were divided into two sections. The first section was fixed in neutral buffered formalin (10%) for histological examinations. The second section was employed for biochemical detection of COX-2, PGE2, TNF-α, and NF-kB using Enzyme-linked immunosorbent assay (ELISA) technique. Furthermore, the gastric contents were preserved for evaluation of pepsin level in gastric juice.

Following the manufacturer's protocol (MyBioSource®, Inc. San Diego, USA), rat ELISA kits were employed for the assessment of pepsin (Cat # MBS2507364) in gastric contents, COX-2 (Cat # MBS266603), PGE2 (Cat # MBS730592), TNF-α (Cat # MBS175904), and NF-κB (Cat # MBS453975) in homogenates of gastric mucosal tissue.

2.5. Histopathological assessment of inflammation in gastric mucosal tissue

The fixed stomach samples (10% buffered formol-saline) were cut into 4 mm paraffin slices and stained with hematoxylin and eosin. The tissue slides were then inspected using a Leica microscope (Leica Microsystems GmbH®, Wetzlar, Germany). All light microscopic exams and morphometric data were analyzed using the Leica Application module, which was linked to a complete HD microscopic imaging system (Leica Microsystems GmbH®, Germany). (22).

2.6. Collection of gastric mucosal tissue for biochemical analysis

Before indomethacin was given to any of the animals, pyloric ligation was performed in order to collect gastric juice. A midline incision was made in the abdomen while the rats were under anesthesia. The pyloric part of the stomach was carefully mobilized and ligated with a silk ligature around the pyloric sphincter. After the animals were given time to recover from anesthesia, the abdominal incision was sutured.

The second portion of the stomach in each specimen was preserved at a temperature of -80°C. Following this, the gastric mucosa was scraped and subsequently homogenized in a solution of 2 ml normal saline containing 0.1 M dithiothreitol. The resulting mixture was then centrifuged at

2000 g for a duration of 10 minutes at room temperature. The ELISA technique was employed to determine COX-2, PGE2, TNF-α, and NF-κB in the supernatant (23).

2.7. Collection of gastric juice for measurement of pepsin level using ELISA technique

The stomach contents were centrifuged at 3000 rpm for 10 minutes, and the volume of each sample was calculated as the volume of gastric juice. Each gastric juice sample was first diluted 1:100 with N/100 HCl. Each 1 ml of diluted juice was mixed with 5 ml of 2% bovine serum albumin solution. In a water bath, the mixture was incubated for precisely 10 min at 37ºC. After incubation, 10 ml of 0.3 M trichloroacetic acid was added and the mixture was heated for 5 min. After that, the solution was centrifuged for 5 min at 3000 rpm and filtered. Each 1 ml of the filtrate was mixed with 2 ml of 0.5 N NaOH and 0.2 ml of Folin reagent. Colorimetrically, the color that formed after 20 min was measured at 680 nm (24, 25).

2.8. Evaluation of cyclooxygenase-2 (COX-2) in gastric tissue using ELISA technique

The assessment of cyclooxygenase-2 (COX-2) in gastric tissue was performed using the corresponding rat ELISA kit provided by the manufacturer (MyBioSource®, Inc. San Diego, USA) (26).

2.9. Detection of prostaglandin E2 (PGE2) in gastric mucosa by ELISA technique

ELISA kits were utilized for measurement of gastric (stomach tissue homogenate supernatant) content of PGE2 following the protocol provided by the manufacturer (MyBioSource®, Inc. San Diego, USA) (27).

2.10. Analysis of the pro-inflammatory mediator, Tumor Necrosis Factor Alpha (TNF-α), using ELISA technique

The level of the pro-inflammatory mediator, Tumor Necrosis Factor Alpha (TNF-α) in the gastric tissue homogenates were measured using ELISA kits obtained from (MyBioSource®, Inc. San Diego, USA). Measurement of marker absorbance was performed at a wavelength of 450 nm (26, 28).

2.11. Assessment of the inflammatory mediator, Nuclear Factor-KB (NF-κβ), using ELISA technique

The levels of inflammatory markers Nuclear Factor-KB (NF-κβ) in the gastric tissue homogenates were measured using ELISA kits obtained from (MyBioSource®, Inc. San Diego, USA). Measurement of marker absorbance was performed at a wavelength of 450 nm (27).

2.12. Data and statistical analysis

Only groups of at least five animals were included for statistical analysis. The current study used one-way ANOVA to determine statistical significance. Tukey's multiple comparisons test was also used to assess differences between groups. For statistical significance, a p-value of less than 0.05 was used. Averages and standard deviations (SDs) are shown for all numbers. GraphPad Prism® program for Windows (Version 9.2.0.332-San Diego, USA) was used for all data administration and analysis.

3. Results

3.1. Empagliflozin (Empa) attenuates peptic ulcers induced by indomethacin (Indo) in rats

First, the occurrence of PU was confirmed as a result of a single oral gavage of Indo (100 mg/kg). As shown in **Figure 4B**, multifocal ulcerative areas were detected in the glandular mucosa which is characterized by desquamation of the epithelial lining and mixed with hemorrhages and accumulation of eosinophilic and karyorrhectic necrotic tissue debris. Several cases showed excessive inflammatory cell infiltration in the mucosa and submucosal layers. Increased vascular permeability was observed in the submucosal blood vessels with subsequent abundant edema. Fortunately, Empa shows marked protective / anti-ulcerative actions. The glandular mucosa revealed apparently normal gastric epithelial cells. However, some examined sections showed submucosal edema and hyperemic blood vessels (**Figure 4C**).

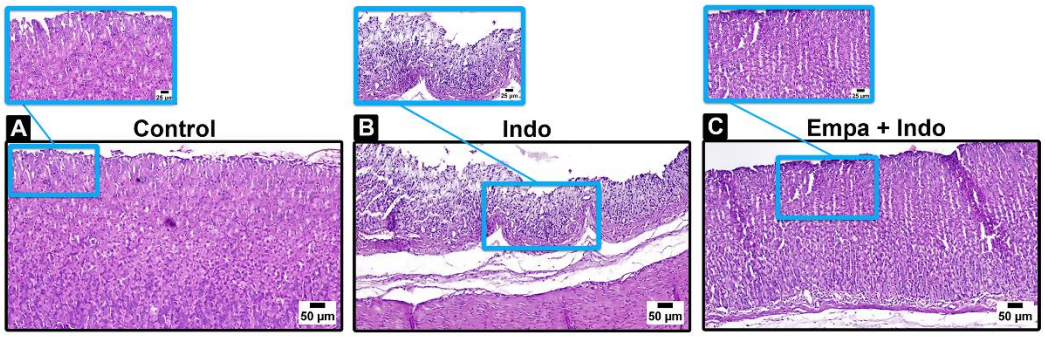

Figure (4): Effect of empagliflozin (Empa) on the cellular architecture of gastric mucosal tissue using histopathological examination

(**A**), cross sections show normal mucosa with intact epithelial lining. (**B)**, multifocal ulcerative areas were detected in the glandular mucosa which is characterized by desquamation of the epithelial lining and mixed with hemorrhages and accumulation of eosinophilic and karyorrhectic necrotic tissue debris. Several cases showed excessive inflammatory cell infiltration in the mucosa and submucosal layers. Increased vascular permeability was observed in the submucosal blood vessels with subsequent abundant edema. (**C**), Empa shows marked protective / anti-ulcerative actions. The glandular mucosa revealed apparently normal gastric epithelial cells. However, some examined sections showed submucosal edema and hyperemic blood vessels. The data are provided as mean ± SEM (n = 5). (Scale bar = 50 µm & 25 µm).

3.2. Empagliflozin (Empa) abrogates pepsin in gastric juice of indomethacin-induced peptic ulcer

For confirmatory purposes, pepsin contents were measured. Several reports proved that pepsin is involved in the progression of ulcerations and laryngopharyngeal reflux (29, 30, 31). In the Indo group, there was a significant elevation in pepsin activity by (**5.4 times**) compared to the control group. Empa causes a significant fall in pepsin activity by (**50%**) as compared to the indomethacin group (**Figure 5**).

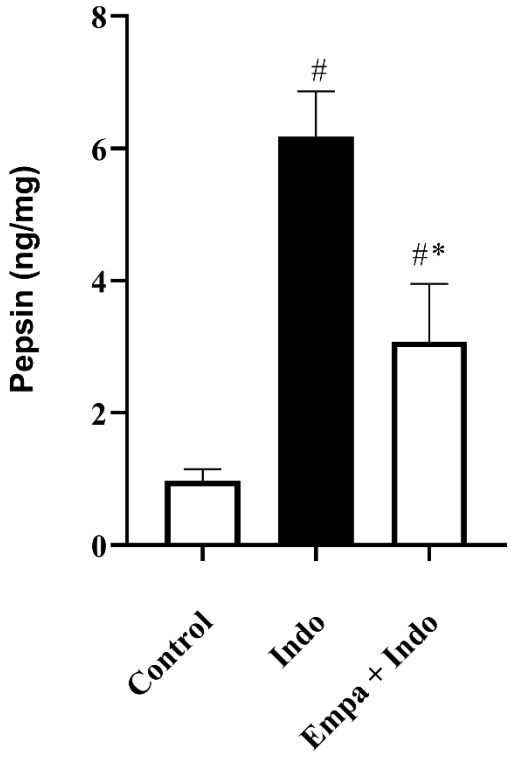

Figure (5): Effect of empagliflozin (Empa) on pepsin level in gastric juice in indomethacin-induced peptic ulcer in gastric mucosa of indomethacin (Indo)-induced peptic ulcer using ELISA technique

Total stomach extract from rats treated with either the isotonic saline (**Control**) or a single oral gavage dose of indomethacin (**Indo**) (100 mg/kg) or empagliflozin (10 mg/kg; p.o for 14 days) and 1 hr. before receiving of a single oral gavage of indomethacin (**Indo**) (100 mg/kg) (**Empa + Indo**)

Data represented the mean ± SD.

significantly different from the control group at $p<0.05$

#* significantly different from the indomethacin and control group at $p<0.05$

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test.

3.3. Empagliflozin restores the normal content of cyclooxygenase-2 (COX-2) in gastric mucosa of indomethacin-induced peptic ulcer

Although Indo is a non-selective inhibitor of COX, however, higher doses of Indo are associated with lose of its therapeutic efficacy and a high incidence of toxic actions (32). As shown in the indomethacin (Indo) group (**Figure 6**), there was a significant decrease in COX-2 level by (**83%**) as compared to the control group. The co-administration of empagliflozin (Empa) causes a significant increase in COX-2 level by (**136%**) compared to the indomethacin group.

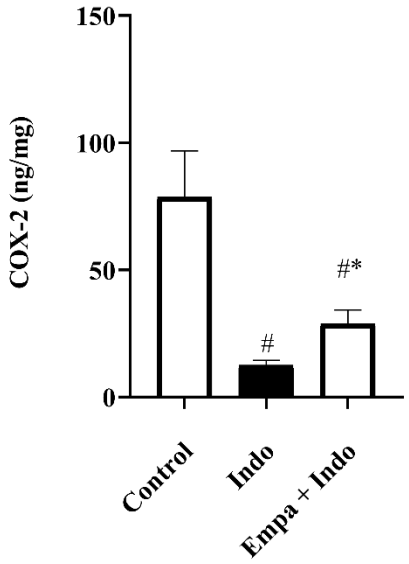

Figure (6): Effect of empagliflozin (Empa) on cyclooxygenase (COX-2) in gastric mucosa of indomethacin (Indo)-induced peptic ulcer using ELISA technique

Total stomach extract from rats treated with either the isotonic saline (**Control**) or a single oral gavage dose of indomethacin (**Indo**) (100 mg/kg) or empagliflozin (10 mg/kg; p.o for 14 days) and 1 hr. before receiving of a single oral gavage of indomethacin (**Indo**) (100 mg/kg) (**Empa + Indo**)

Data represented the mean ± SD.

significantly different from the control group at $p<0.05$

#* significantly different from the indomethacin and control group at $p<0.05$

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test.

3.4. Empagliflozin replenishes the normal levels of the protective prostaglandin E-2 (PGE2) in gastric mucosa of indomethacin-induced peptic ulcer

It is well established that prostaglandin E2 (PGE2) is crucial for gastroprotection of stomach walls (33). As demonstrated in **Figure 7**, the synthesis of mucosal PGE2 was markedly decreased in Indo group by (**56%**) compared to that in the control group. However, the mucosal synthesis of PGE2 in rats, co-treated with Empa was significantly increased by (**71%**) compared to that of Indo group.

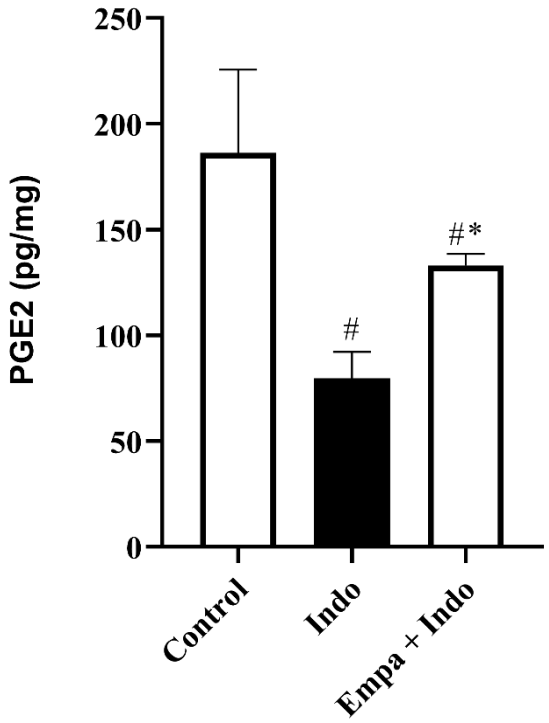

Figure (7): Effect of empagliflozin (Empa) on prostaglandin E2 (PGE2) content in gastric mucosa of indomethacin (Indo)-induced peptic ulcer using ELISA technique

Total stomach extract from rats treated with either the isotonic saline (**Control**) or a single oral gavage dose of indomethacin (**Indo**) (100 mg/kg) or empagliflozin (10 mg/kg; p.o for 14 days) and 1 hr. before receiving of a single oral gavage of indomethacin (**Indo**) (100 mg/kg) (**Empa + Indo**)

Data represented the mean ± SD.

significantly different from the control group at $p<0.05$

#* significantly different from the indomethacin and control group at $p<0.05$

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test.

3.5. Empagliflozin inhibits the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) in gastric tissue of indomethacin-induced peptic ulcer

Compared with the control group, the level of TNF-α was significantly increased in Indo group by (**2.5 times**). Interestingly, Empa causes a significant suppression effect on the level of TNF-α by (**55%**) when compared to Indo group (**Figure 8**).

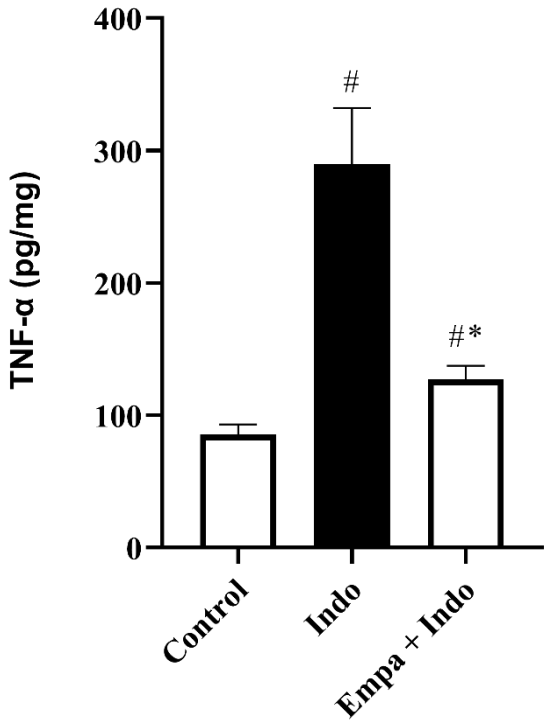

Figure (8): Effect of empagliflozin (Empa) on serum level of proinflammatory cytokine tumor necrosis factor-alpha (TNFα) in gastric mucosa of indomethacin (Indo)-induced peptic ulcer using ELISA technique

Total stomach extract from rats treated with either the isotonic saline (**Control**) or a single oral gavage dose of indomethacin (**Indo**) (100 mg/kg) or empagliflozin (10 mg/kg; p.o for 14 days) and 1 hr. before receiving of a single oral gavage of indomethacin (**Indo**) (100 mg/kg) (**Empa + Indo**)

Data represented the mean ± SD.

significantly different from the control group at $p<0.05$

#* significantly different from the indomethacin and control group at $p<0.05$

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test.

3.6. Empagliflozin attenuates the inflammatory nuclear factor-KB (NF-κB) activity in gastric tissue of indomethacin-induced peptic ulcer

In Indomethacin group, NF-κB activity was significantly increased by (**6 times**) compared with the control while treatment with Empagliflozin cause a significant decrease in activity by (**53%**) compared to indomethacin-treated group (**Figure 9**).

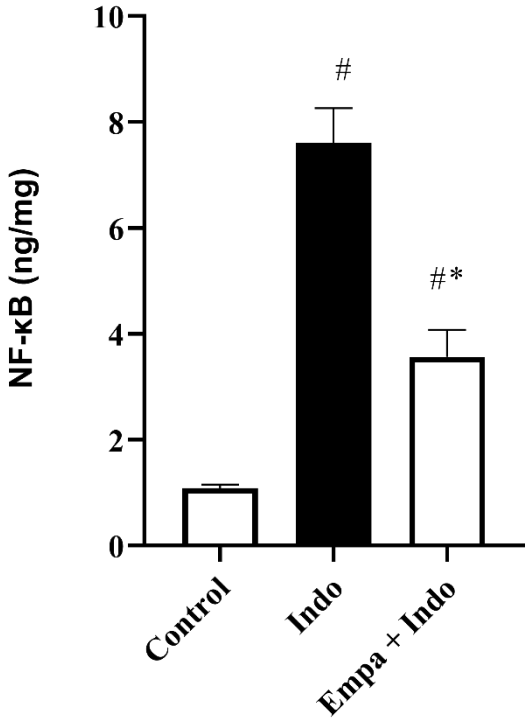

Figure (9): Effect of empagliflozin (Empa) on nuclear factor-κB (NF-κB) activity in gastric tissue of indomethacin (Indo)-induced peptic ulcer using ELISA technique

Total stomach extract from rats treated with either the isotonic saline (**Control**) or a single oral gavage dose of indomethacin (**Indo**) (100 mg/kg) or empagliflozin (10 mg/kg; p.o for 14 days) and 1 hr. before receiving of a single oral gavage of indomethacin (**Indo**) (100 mg/kg) (**Empa + Indo**)

Data represented the mean ± SD.

significantly different from the control group at $p<0.05$

#* significantly different from the indomethacin and control group at $p<0.05$

Statistical analysis was carried out by one-way ANOVA followed by Tukey-Kramer as a post-hoc test.

4. Discussion

PU is a term used to describe a series of chronic symptoms that damage the mucosal integrity of the stomach and/or duodenum lining, and it is still a frequent source of gastrointestinal morbidity and death (34). Epigastric discomfort, perforations, bloating, nausea, blood in the stool or vomit,

lack of appetite, and weight loss are common symptoms (35). The global prevalence of PU is estimated to be 200-250 per 100,000 people (36). PU affects both sexes equally and may begin at any age, although the most common age is between the ages of 10-15 years (37). An imbalance in the gastric mucosa between defensive factors (i.e., decreased bicarbonate production, prostaglandins (PGs), nitric oxide (NO), and anti-oxidants) and aggressive factors (free radicals, excess secretion of gastric acid and pepsin) is reported to be a fundamental mechanism involved in the disease's pathogenesis (38). Infection with Helicobacter pylori, alcohol use, Zollinger-Ellison syndrome, and use of NSAIDs are all risk factors for developing peptic ulcers (34).

In the treatment of both short-term and long-term pain, NSAIDs like indomethacin (Indo) are often used. Rheumatoid arthritis, degenerative joint disease, gout, acute musculoskeletal dysfunction, inflammation, and edema were among the first conditions for which it was approved in 1963. Strong anti-inflammatory, pain-relieving, and fever-reducing actions characterize Indo (39). However, the severity and frequency of its adverse effects have severely limited its therapeutic use. Many experimental animals show multisystem lesions from Indo, including damage to the kidney, liver, gut, brain, lungs, spleen, blood vessels, and glands (40, 41). Interestingly, the mechanism by which long-term Indo exposure produces multi-organ toxicity is not fully known and has not been thoroughly explored. Inhibition of prostaglandin production and cellular respiration, DNA damage, and an increase in oxidative stress are thought to be implicated in the etiology of Indo-induced multi-organ toxicities (40, 42, 43).

It is a widely accepted fact that Indo effectively inhibits two isoforms of COX, namely COX-1 and COX-2. The biosynthesis of PGE2 is facilitated by two COX isoforms, which are considered pivotal enzymes. The COX-1 isoform is ubiquitously expressed in various tissues, including the gastrointestinal tract, and is responsible for PGE2 production. In contrast to its ubiquitous expression in most tissues, COX-2 exhibits negligible or minimal expression but is swiftly induced in the context of inflammation. As PU represents a primary characteristic of both inflammation and gastritis, there is a significant reduction in the defensive PGE2 synthesis. Accordingly, Indo may induce PU due to attenuating the gastroprotective layer (44, 45).

In consistent with these findings, the present study validates the existence of PU by means of inhibiting two COX isoforms, leading to a decrease in the gastroprotective PGE2 layer, thus corroborating the aforementioned observations. PGE2 represent the primary metabolites of

arachidonic acid. Generally, PGE2 has been observed to exert regulatory control over the production of gastric mucus and bicarbonate, as well as the reduction of acid output. Additionally, PGE2 has been found to facilitate the restoration of the gastric mucosa through the dilation of vessels, improvement of mucosal blood flow, and acceleration of mucosal healing (44, 45). The primary aim of this investigation is to repurpose Empa as a COX-2 inhibitor, thereby preserving COX-1 and reducing the inhibitory effect on PGE2. Several studies proved that Indo can induce PU via reduction of two COX isoforms, PGE2 concurrently with elevation of pepsin and inflammatory mediators (i.e., TNF-α and NF-κβ) (1, 10, 46, 47). The current findings also agree with these observations.

On the other hand, Empa is a promising anti-inflammatory candidate for the treatment of PU induced by Indo. Furthermore, the high mucin production was evidenced to alleviate PU in Empa-treated rats (48). Interestingly, the combination therapy of Emp plus Baicalein was proved to attenuate gastric ulcers in rats via modulation of HO-1/SIRT1 / HMGB1 signaling pathway (49). Recently, N. Lee *et al.*, (50) proved that Empa downregulates mRNA expression of COX-2, TNF-α and NF-κβ in lipopolysaccharides-induced macrophages. Furthermore, Z. H. Maayah *et al.*, (20) confirmed a significant reduction of IL-5, IL-6, IL-16, IL-17, and TNF-α in acute septic renal injury. Regardless its anti-diabetic action, C. A. Alvarez *et al.*, (13) demonstrated that Empa can enhance cardiac outcomes in non-diabetic models of heart failure by decreasing the activation of cardiac inflammation. This indicates that the therapeutic advantages of empagliflozin may not be solely attributed to its effects on diabetes. The present findings adhere to these observations. Therefore, it is possible that Empa helps reduce Indo-induced PU, principally by lowering inflammation of the stomach lining.

Interestingly, Empa can effectively reduce gastric contents of pepsin. In fact, gastric juice is made up of water, mucus, hydrochloric acid, pepsin, and intrinsic factor. Pepsin is the major enzyme involved in protein degradation among the five components. Proteins are hydrolyzed into smaller peptides and amino acids, which aids in their absorption in the small intestine. The stomach lining's primary cells are in charge of secreting pepsinogen, an inactive version of the enzyme pepsin. The stomach uses this mechanism to prevent the self-digestion of protective proteins found in the gastrointestinal tract's mucosal lining. An acidic environment is required for pepsin activation. This is owing to the fact that pepsin is originally released as a zymogen.

Hydrochloric acid (HCl) is an essential component of gastric juice that plays a critical role in creating the pH required for efficient pepsin action. When pepsinogen and hydrochloric acid coexist in gastric juice, pepsin is activated (31). Accordingly, Empa can restore the normal balance between defensive factors (i.e., increased PGE2) and aggressive factors (i.e., decreased pepsin).

5. Conclusion

In summary, Empa can alleviate PU induced by Indo through the reduction of COX-2, pepsin, TNF-α, and NF-κβ and elevating gastroprotective PGE2 (**Figure 10**).

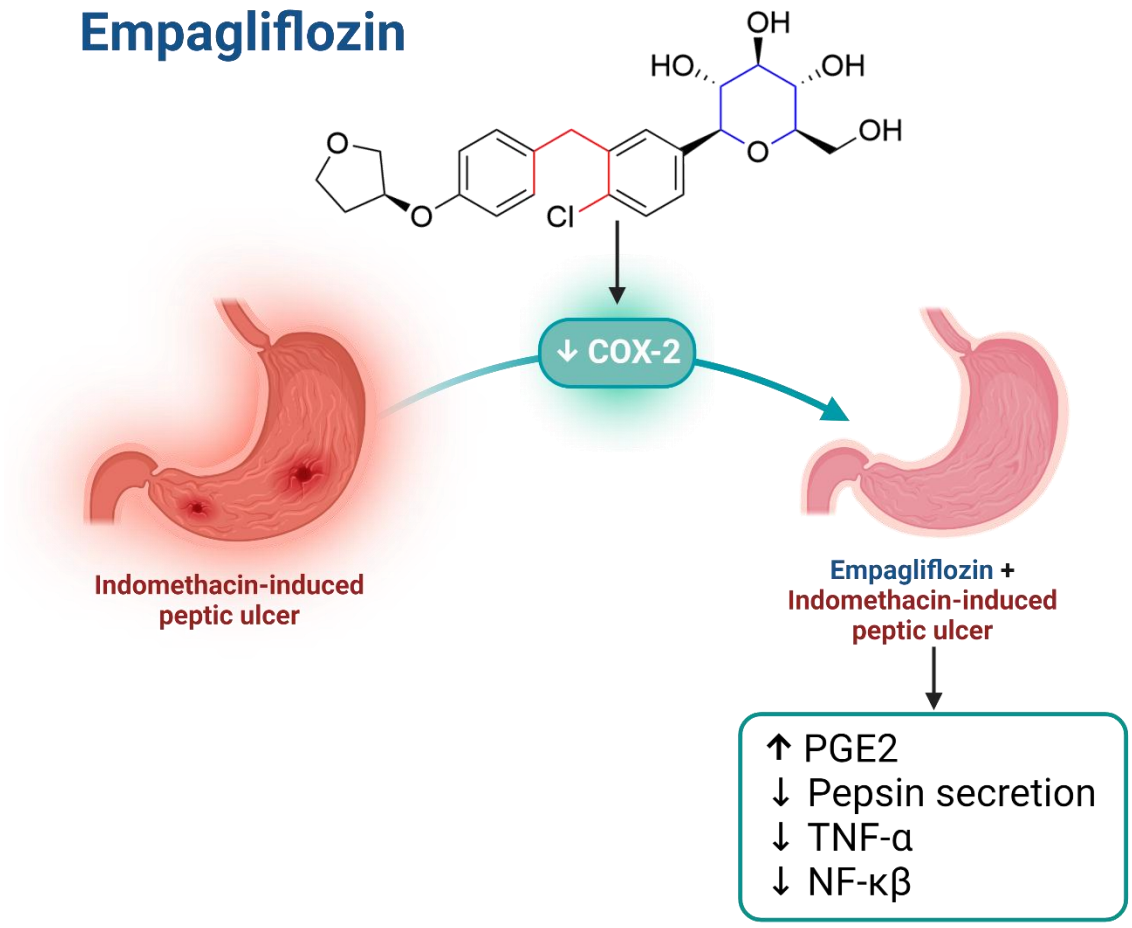

Figure (10): Graphical abstract showing the collective gastroprotective and anti-ulcerative actions of empagliflozin (Empa):

Acknowledgement

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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