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Protective Effects of Infliximab on Indomethacin-Induced Gastric Ulcer in Rats: The Role of MAPK/NF-KB p65 Signaling Pathway

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Abstract: Nearly 25% of gastric ulcer (GU) instances globally are induced via nonsteroidal anti-inflammatory drugs (NSAIDs). Infliximab (Inflix), a monoclonal antibody, is widely utilized to medicate autoimmune disorders and chronic inflammatory disorders. This investigation aimed to determine if Inflix could prevent indomethacin (IND)-induced GU while examining potential protective mechanisms, such as the NF-κB/MAPK signaling pathway. Male albino rats received Inflix (5 and 7 mg/kg, intraperitoneally) on the fifth day, fasted for 24 h, and were exposed to IND (100 mg/kg, orally) for ulcer induction on the eighth day. The study showed that IND caused GU in macroscopic and histopathological manifestations. Pretreatment with Inflix improved gastric tissue damage in histopathological and macroscopic manifestations in a dose-dependent manner. Moreover, Inflix pretreatment increased the IND-affected decline in stomach prostaglandin E2 (PGE2) levels. Additionally, Inflix exerted antioxidant, anti-inflammatory, and anti-apoptotic effects by lowering oxidative stress markers, pro-inflammatory markers, and apoptotic markers in a dose-dependent approach. It was demonstrated that Inflix reduced the mitogen-activated protein kinase (MAPK) and the nuclear factor kappa B (NF-κB) p65 induced by IND-induced GU**.** Inflix exerts a gastro-protective effect via antioxidant, anti-inflammatory, and anti-apoptotic actions, which could be associated with modulating the NF-κB and MAPK cascades.

Keywords: Gastric ulcer, Indomethacin, Infliximab, TNF-α, NF-κB, MAPK

1. INTRODUCTION

Gastric ulcer (GU) represents a popular digestive syndrome that refers to the presence of ulcers in the stomach [1,2]. One of the most frequent causes of ulcer development is the improper utilization of nonsteroidal anti-inflammatory drugs (NSAIDs) [3]. NSAIDs are non-selective cyclooxygenase enzyme inhibitors that hinder cyclooxygenase enzymes 1 (COX-1) and 2 (COX-2) and have antipyretic, analgesic, and anti-inflammatory impacts [4, 5]. Among the frequently utilized NSAIDs, indomethacin (IND) has a potent ulcerogenic possibility in humans [6, 7, 8]. It is well acknowledged that IND administration probably increases the reactive oxygen species (ROS) [9, 10] causing oxidative stress that damage the gastric mucosa, which results in GU [9, 16, 17]. Superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals' generation, all of these factors induce lipid peroxidation [3, 11]. Malondialdehyde (MDA) is one of the crucial byproducts of membrane lipid peroxidation [12, 13], whereas superoxide dismutase (SOD) acts a critical anti-oxidative function that dismutases superoxide radicals into harmless molecules (O_2) [14, 15].

Pro-inflammatory cytokine release may be triggered by IND, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), resulting from the epithelial barrier's rupture [18,19]. Pro-apoptotic mediators are also activated via IND administration. IND can also impede the healing of GU by inhibiting prostaglandin production, particularly PGE2 [21], which has gastroprotective anti-inflammatory properties. Caspases including caspase-3 (Casp-3) are pro-apoptotic proteolytic enzymes that are either activated by the intrinsic mitochondrial system or by the extrinsic death receptor pathways, mediating apoptotic cell death [8, 16]. MAPKs are major

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intracellular signal transduction pathways [17, 20]. P38, c-jun-n-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK), are the active subunits from the MAPKs activation [22-26.] The stimulated MAPK cascade activates the NF-κB cascade [12]. Moreover, NF-kB reacts immediately to stress and inflammatory responses and is crucial for the pathogenesis of GU [27]. ROS and pro-inflammatory chemicals are created either directly or through the activation of the MAPKs/NF-κB cascade [28, 29.] TNF-α, IL-1β and IL-6, are the products of the MAPKs/NF-κB signaling transduction cascade[12, 27, 30].

Infliximab (Inflix) is a monoclonal antibody that is used therapeutically to treat Crohn's disease (CD), rheumatoid arthritis (RA), and dermatological diseases [25, 33, 34]. It was mentioned to scavenges oxygen free radicals and hinder inflammation by its modulating effect on the TNF-α receptor [35, 36.] Additionally, Inflix may prevent inflammation via the MAPK/ NF-κB signaling pathway [20, 25]. It is thought that TNF-α binds to its receptor and activates several signaling pathways, including JNK, MAPK, ERK, and NF-κB. After that, many transcription factors will be activated, resulting in the various proteins' transcription related to the inflammatory reactions and other consequences [37-42.]

No beneficial strategies are available for complete ulcer healing [31]. The most effective choice to protect against NSAID-related GU toxicity is either the combination of NSAIDs and proton pump inhibitors (PPIs) [31] or the selective COX-2 inhibitors' choice [32]. There are numerous severe side effects related to the long-term utilization of anti-ulcer medications. Gastric ulcer patients frequently stop using the medication due to adverse effects. As a result, patients require novel therapies [8].We hypothesized that Inflix might attenuate IND-induced GU by preventing TNF-α induced inflammations by regulating the MAPK/NF-κB signaling cascade.

2. METHODS

2.1. Animals

The Nile Company for Pharmaceuticals and Chemical Industries' animal facility provided forty adult male albino rats considering 180 and 200 g (Cairo, Egypt). The experimental animals received frequent feedings and free access to water while maintaining 55% humidity, a constant temperature of 25 ± 2 °C, and a 12-hour light/dark cycle. Before any experimental procedures, animals had a one-week acclimatization period. The research methods were done in line with the Animal Ethics Committee's recommendations of the Faculty of Pharmacy at Al-Azhar University in Egypt and the eighth edition of the NRC Instructions for the Care and Use of Laboratory Animals (permission number: 203/2019).

2.2. Medicines and chemicals

The chemicals were obtained from Sigma-Aldrich and were of highly analytical quality (St. Louis, MO, USA). The pharmaceutical and chemical sectors (El-Nile Co., Cairo, Egypt) provided vials of IND (Liometacen®), which contained the active ingredient meglumine (77.2 mg) and was equivalent to 50 mg of IND. For the pharmaceutical and chemical industries, esomeprazole (Esmorap®) vials containing esomeprazole 40 mg sodium salt were gotten from AUG Pharma (Cairo, Egypt). Infliximab (Remicade®) was provided as vials (infliximab 100 mg powder for concentrate) from Schering-Plough Labo for the pharmaceutical and chemical industries (Cairo, Egypt).

2.3. Ulcer induction

Animals have fasted for 24 hours with free access to water. Gastric ulceration of rats was induced by giving a single oral dose of indomethacin IND (100mg/kg). Gastric ulceration was detected after 6 hrs of IND administration [43, 44].

Experimental design

Fourty male albino rats weighing (180g - 200g) divided into 5 groups (eight per group) and regimen treatments as follow below and demonstrated in Figure 1:

Group I: rats received saline (1ml/kg/daily, p.o.) from 1st day until the end of experiment (8 days) as control-group.

Group II: rats received saline (1ml/kg/daily, p.o.) from 1st day until the 7th day. They fasted first for 24 hrs before IND administration with access to water. In the day of scarification (the 8th), rats received IND (100mg/kg, p.o.) and after 6 hrs, they were killed for examination as IND group[45].

Group III: rats received Esmo (50mg/kg/daily,i.p.) as Esmo group [20] from 1st day until the 7th day. In the 7th day, they fasted for 24 hrs then received IND (100mg/kg, p.o.).

Group VI: rats received Inflix 5mg/kg (i.p.) as Inflix 5mg group only in the 5th day of the experiment [46]. In the 7th day, they fasted for 24 hrs then received IND (100mg/kg, p.o.).

Group V: rats received Inflix 7mg/kg (i.p.) as Inflix 7mg group only in the 5th day of the experiment [46]. In the 7th day, they fasted for 24 hrs then received IND (100mg/kg, p.o.).

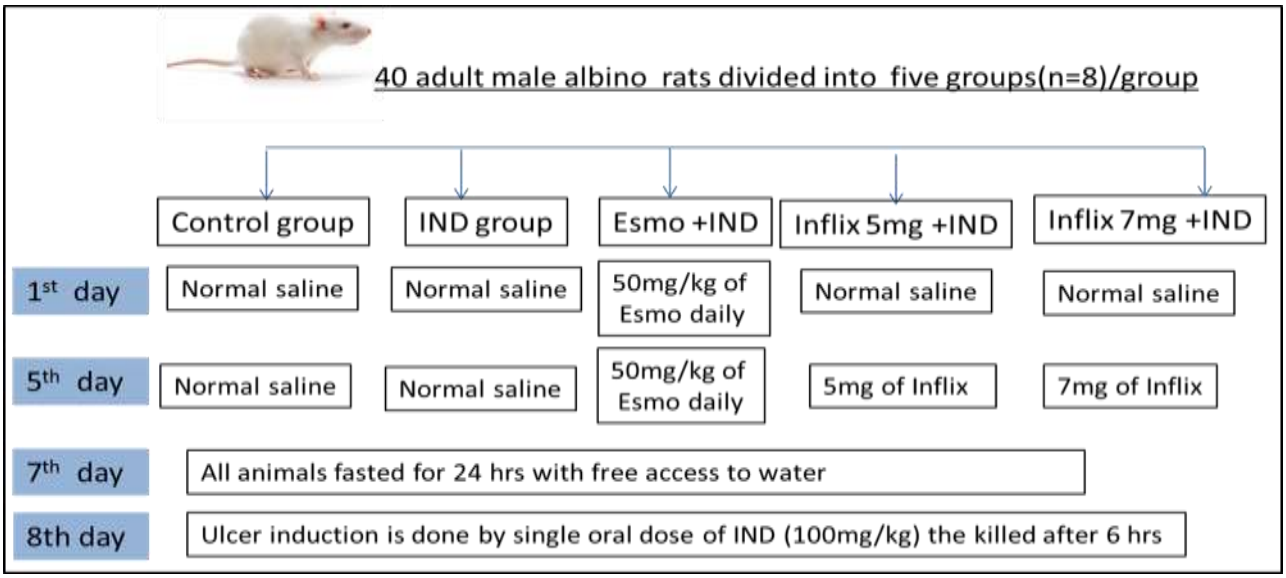

Figure 1: Schematic of study design.

To assess serum TNF-α, IL-1β, and IL-6 level, blood specimens were taken through retro-orbital sinus puncture under mild ether anesthesia six hours after the ulcer was induced. After the animals were killed, the stomach tissues of the rats were removed, cleaned with saline, and examined under a microscope. The stomach tissues were then weighed. For histological investigation, two distinct stomach tissues were embedded in a 10% formalin solution. 10% (w/v) tissue homogenates in ice-cold phosphate-buffered saline were made from three stomach tissues for biochemical analysis. The centrifugation of homogenates occurred at 4 °C for 15 min at 4000 rpm. Until the oxidative stress markers, pro-inflammatory biomarkers, and PGE2 examination, the acquired supernatant was preserved at −80 °C. Further three stomach tissues were kept at − 80 °C and homogenized in lysis buffer for a western blot assay of apoptotic markers (BAX, BCL-2, and casp-3), the MAPK pathway (p-MAPK, p-ERK, and p-JNK), and p-NF-κB P65 cascade.

2.4. Macroscopic evaluation

The stomachs were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a 10X magnifier lens to assess the formation of ulcers. The numbers of ulcers were counted. Scoring of ulcer will be made as follows [48]:

Normal colored stomach..... (0)
Red coloration..... (0.5)
Spot ulcer.....…. (1)
Hemorrhagic streak... (1.5)
Deep Ulcers.....… (2)
Perforation.....… (3)

2.5. Histopathological evaluation

Specimens of the stomach were embedded in paraffin, fixed in 10% formalin, and stained with hematoxylin and eosin after being sectioned into 4 µm thick pieces [49].

2.6. Biochemical parameters

2.6.1. Determination of oxidative stress biomarkers

Utilizing kits purchased from Biodiagnostic Co., Egypt, stomach homogenates were utilized to estimate the lipid peroxides' number generated as SOD, total antioxidant capacity (TAC), and MDA. The stages were done per the manufacturer's guidelines, and the parameters were calculated according to the guidelines.

2.6.2. Determination of pro-inflammatory biomarkers

An ELISA kit (CUSABIO, USA) was utilized to determine the IL-6, IL-1β, and TNF-α level in the stomach and serum. The manufacturer's protocol was followed in accordance with the guidelines for each parameter that was tested, and concentration samples were measured per the manufacturer's instructions.

2.6.3. Determination of gastric PGE2

Using an ELISA kit purchased, gastric PGE2 was measured (CUSABIO, USA). The manufacturer's protocol was implemented in accordance with the guidelines for each parameter that was tested, and concentration samples were measured in line with the manufacturer's guidelines.

2.6.4. Western blot analysis for determination of gastric apoptotic markers (BAX, BCL-2, and casp-3) and MAPK and NF-κB P65 pathways

The stomach tissue was homogenised in a lysis solution involving 50 mM Tris-Cl, 0.5% Triton X-100, 300 mM NaCl, and a protease inhibitor, which also had a pH of 7.5. After that, the buffer was incubated for 30 minutes at 4 °C. At 4 °C, the lysates were spun for 20 minutes at 15,000 rpm. 30 g of proteins were put onto a 10% SDS/PAGE to evaluate the protein concentrations utilising the Bradford protein assay reagent (Bio-Rad, USA). The membrane was rinsed twice in TBS-0.05% Tween-20 (TBS-T) before being blocked for an hour in 5% skimmed milk powder after the gels had been transferred to nitrocellulose membranes and each antibody had been applied. Then, primary antibodies for apoptotic markers for BAX, BCL-2, and casp-3 and P-MAPK, P-ERK, P-JNK, and P-NF-κB P65 were incubated overnight on the membranes (1:2000 Cell Signaling Technology) at 4 °C. The membrane was then exposed to an anti-mouse secondary antibody at 37 °C for one hour and rinsed five times for five minutes in TBS-T (dilution 1:5000, CST, USA). The blot was TBST-washed three to five times for five minutes. The chemiluminescent substrate (ClarityTM Western ECL substrate) was incorporated to the blot in line with the manufacturer's instructions. Using a CCD camera-based imager, the chemiluminescent signals were captured. After protein normalisation on the ChemiDoc MP imager, the target proteins' band intensities were contrasted with the control specimen's β-actin utilising image analysis software.

2.7. Statistical analysis

Means ± SEM were used to display the data. The results were statistically analysed utilising one-way ANOVA and post hoc Tukey's multiple comparison assays utilising GraphPad Prism software (version 8). Values of $p \leq 0.05$ and 0.005 were statistically substantial for all analyses.

3. RESULTS

3.1. Macroscopic examination of the stomach

Macroscopic examination of stomach from control group revealed no lesions but there were a presence of slight red coloration. While stomach from IND group revealed the presence of hemorrhagic streaks and deep ulcer. Esmo pretreatment revealed presence of red coloration, spot ulcer and less deep ulcer compared to IND group. Pretreatment with Inflix 5 and 7mg revealed presence of red coloration, spot ulcer and less deep ulcer compared to IND group as demonstrated in Figure 2.

Effect of Inflix on the ulcer score in IND-induced GU in rats

Contrasted to the control group, IND administration made a statistically significant elevation in lesions with a score of 4± 0.37 (Table 1). Pretreatment with Esmo significantly decreased lesions with a score of 1.5± 0.22 contrasted to the IND group. Inflix 5 mg and 7 mg pretreatment significantly decreased lesions with a score of 1.5± 0.22 and 1.33± 0.21, respectively, compared to the IND group.

Table 1: Effect of infliximab (Inflix) on the ulcer score in indomethacin-treated rats

Groups/Parameters	Ulcer score
Control group	0.08±0.08
IND group	$4\pm0.37^{**}$
Esmo +IND	$1.5\pm0.22^{**\#\#}$
Inflix 5mg+IND	$1.5\pm0.22^{**\#\#}$
Inflix 7mg+IND	$1.33\pm0.21^{**\#\#}$

$^{**}p \leq 0.005$, significant difference compared with control $^{\#\#}p \leq 0.005$, significant difference compared to IND Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole and Inflix: infliximab.

3.2. Effect of Inflix on histopathological alterations in IND-induced GU in rats

A histopathology investigation was conducted to further support Inflix's stomach protective function. Rat-control stomach slices stained with H&E revealed normal histopathological structure during microscopic examination (Fig.3.A). IND administration caused focal ulceration with hemorrhage and hemosiderosis in the mucosal layer linked with inflammatory cell infiltration and edema in the underlying submucosal layer (Fig.3.B).

Pretreatment with Esmo resulted in edema with a few inflammatory cells infiltrating the submucosal layer (Fig.3.C). Inflix 5 mg pretreatment showed focal necrosis in the mucosal layer, while the underlying submucosa had edema with inflammatory cell infiltration (Fig.3.D). Inflix 7 mg group pretreatment showed edema in the submucosal layer with focal inflammatory cell infiltration (Fig.3.E).

3.3. Effect of Inflix on gastric oxidative stress biomarkers in IND-induced GU in rats

Ulcer induction by IND significantly elevated MDA by 462% contrasted to the control group and significantly lowered SOD and TAC by 18% and 38%, consecutively, contrasted to the control group (Table 2). Pretreatment with Esmo decreased MDA by 43% compared to IND group and significantly increased SOD and TAC by 322% and 217%, respectively, compared to IND group. Pretreatment with Inflix 5 mg significantly decreased MDA levels by 54% compared to IND group. It significantly

increased SOD and TAC by 521% and 212%, respectively, compared to IND group. Pretreatment with Inflix 7 mg significantly diminished MDA level by 27% contrasted to the control group and considerably increased SOD and TAC by 546% and 231%, respectively, compared to IND.

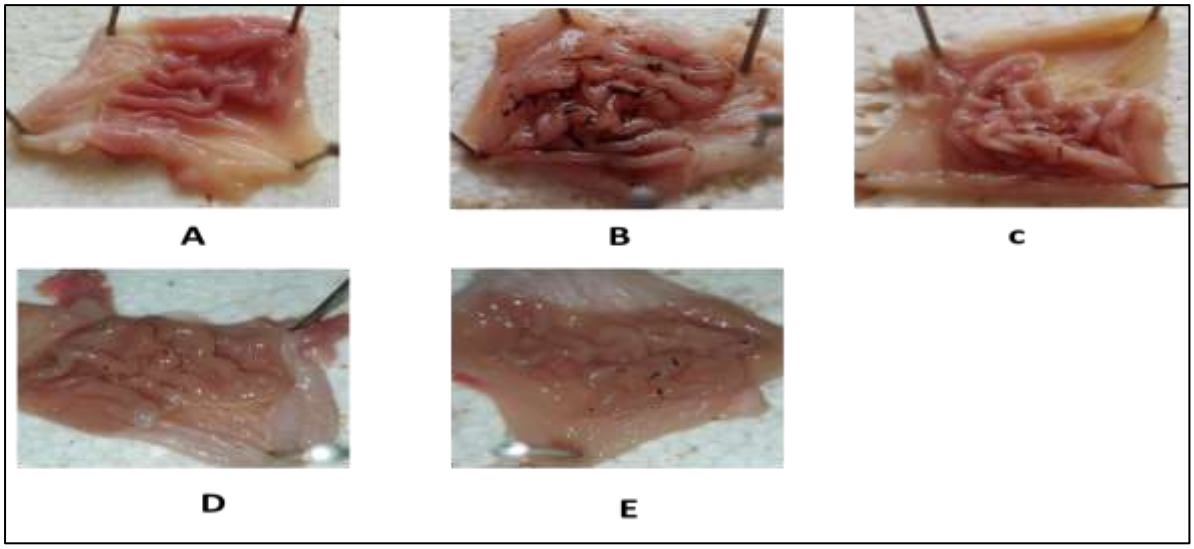

Figure 2: Macroscopic appearance of rat stomach**.**

(A–E) Macroscopic images of the stomach. (A) normal stomach (control), (B) ulcer induced in the stomach using IND,(C) pretreatment of stomach by Esmo 50 mg/kg before IND treatment,(D) pretreatment of stomach by Inflix 5 mg before IND treatment,(E) pretreatment of stomach by Inflix 7mg before IND treatment. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab.

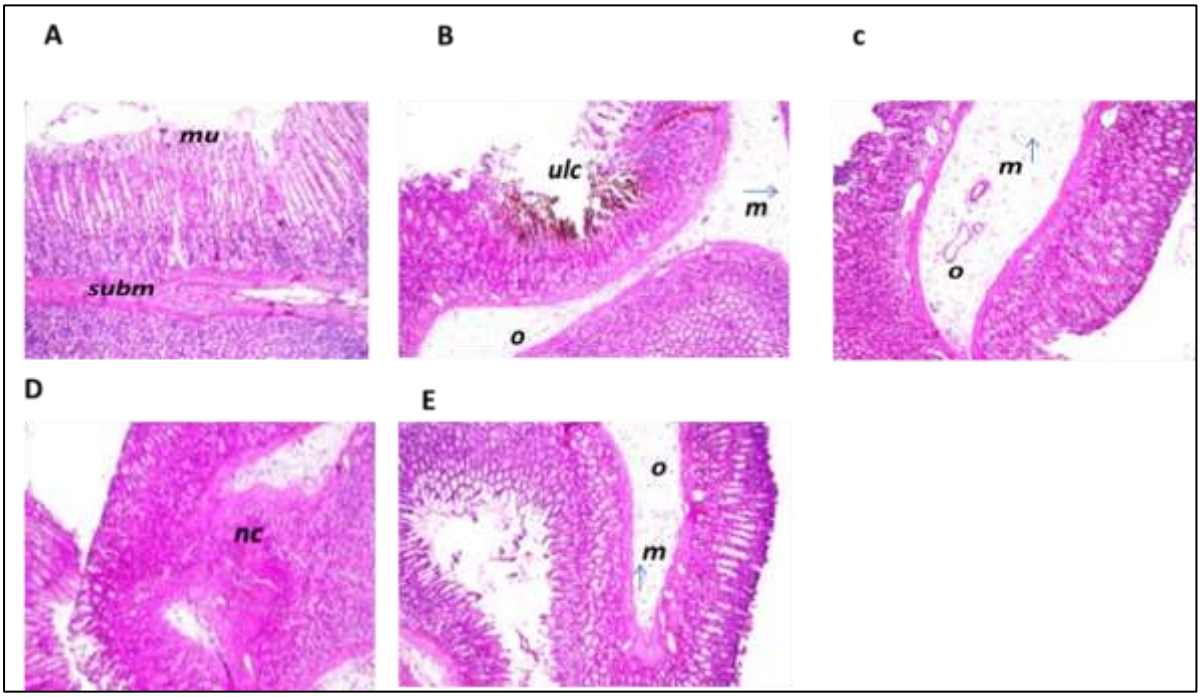

Figure 3: Effect of Inflix on histopathological changes on IND-induced GU in rats

Microscopic examination of H&E-stained stomach sections of control rats showed normal histopathological structure (Fig.3.A). IND administration caused focal ulceration with haemorrhage and hemosiderosis in mucosal layer associated with oedema and inflammatory cells infiltration in the underlying submucosal layer (Fig.3.B).Pretreatment with Esmo Showed oedema with few inflammatory cells infiltration in the submucosal layer (Fig.3.C). Inflix 5mg pretreatment showed Focal necrosis in the mucosal layer while the underlying submucosa had oedema with inflammatory cells infiltration (Fig.3.D). Inflix 7mg group pretreatment showed oedema in the submucosal layer with focal inflammatory cells infiltration (Fig.3.E). Abbreviation: mu : mucosa, subm : submucosa, ulc : ulcer, o: oedema, nc : necrosis, m arrow: inflammatory cell infiltration.

Table 2: Effect of Inflix on gastric SOD, gastric MDA and gastric TAC levels in indomethacin-treated rats

Groups/Parameters	SOD (U/g)	MDA (nmol/g)	TAC activity
Control group	55.52±3.16	27.92±0.41	105.9±1.42
IND group	12.58±0.67**	117.4±1.13**	47.9±1.95**
Esmo group +IND	29.93±1.03$^{**\#\#}$	59.28±2.10$^{**\#\#}$	90.67±2.00$^{**\#\#}$
Inflix 5mg +IND	46.73±1.50$^{*\#\#}$	66.97±2.28$^{**\#\#}$	82.81±2.30$^{**\#\#}$
Inflix 7mg +IND	49.77±2.77$^{\#\#}$	40.98±2.81$^{**\#\#}$	94.8±0.64$^{**\#\#}$

$^{*}p \leq 0.05$, significant difference compared with control, $^{**}p \leq 0.005$, significant difference compared with control, $^{\#\#}p \leq 0.005$, significant difference compared to IND , Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab, SOD: superoxide dismutase, MDA: Malondialdehyde and TAC: Total antioxidant capacity.

3.4. Effect of Inflix on gastric pro-inflammatory biomarkers in IND-induced GU in rats

Ulcer induction by IND significantly elevated gastric TNF-α, IL-1β, and IL-6 via 541%, 256%, and 309%, consecutively, compared to the control group (Fig. 4 A, B, C). Esmo pretreatment substantially reduced gastric TNF-α, IL-1β, and IL-6 by 49%, 56%, and 51%, respectively, compared to IND group. Pretreatment with Inflix 5 mg significantly lowered gastric TNF-α, IL-1β, and IL-6 by 38%, 55%, and 52%, respectively, contrasted to IND group. Inflix 7 mg pretreatment considerably decreased gastric IL-1β, TNF-α, and IL-6 by 36%, 46%, and 40%, consecutively, contrasted to IND group.

3.5. Effect of Inflix on serum pro-inflammatory biomarkers in IND-induced GU in rats

Ulcer induction by IND significantly elevated serum TNF-α, IL-1β, and IL-6 via 613%, 291%, and 376%, consecutively, compared to control group (Fig. 5A, B, C). Pretreatment with Esmo significantly lowered serum TNF-α, IL-1β, and IL-6 via 49%, 49%, and 40%, consecutively, contrasted to IND group. Pretreatment with Inflix 5 mg significantly lowered serum TNF-α, IL-1β, and IL-6 by 42%, 51%, and 54%, consecutively, contrasted to IND group. Pretreatment with Inflix 7 mg significantly reduced serum TNF-α, IL-1β, and IL-6 via 31%, 44%, and 38%, consecutively, contrasted to IND group.

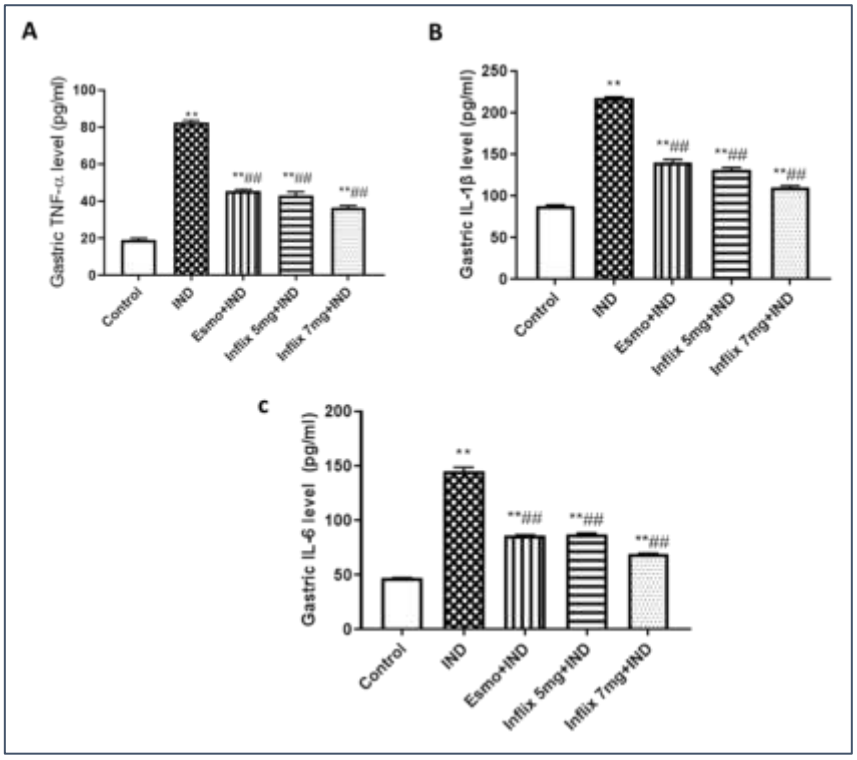

Figure 4: Effect of Inflix on gastric pro-inflammatory biomarkers in on IND-induced GU in rats

$^{**}p \leq 0.005$, significant difference compared with control , $^{\#\#}p \leq 0.005$, significant difference compared to IND, Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab, TNF-α: tumor necrosis factor –alpha, IL-1β: interleukin 1 beta and IL-6: interleukin 6.

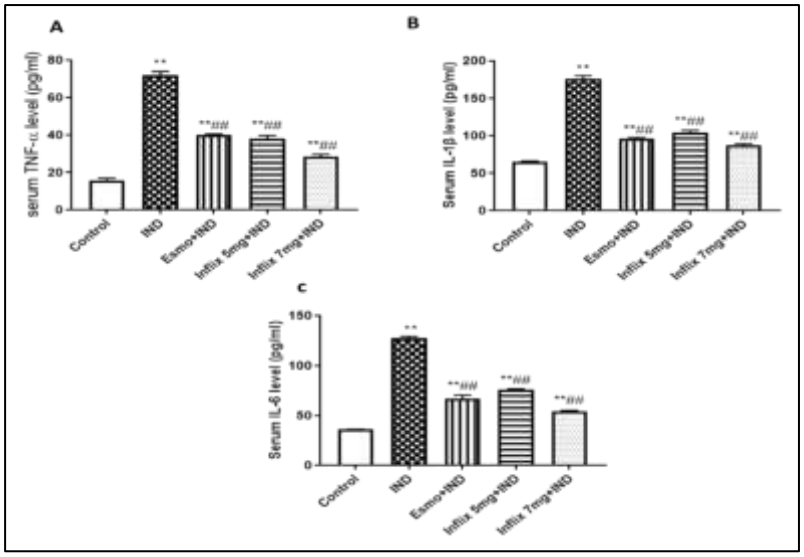

Figure 5: Effect of Inflix on serum pro-inflammatory biomarkers on IND-induced GU in rats

$^{**}p \leq 0.005$, significant difference compared with control, $^{\#\#}p \leq 0.005$, significant difference compared to IND, Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab, TNF-α: tumor necrosis factor –alpha, IL-1β: interleukin 1 beta and IL-6: interleukin 6.

3.6. Effect of Inflix on Gastric PGE2 in IND-induced GU in rats

Concerning the control group, IND significantly lowered the PGE2 level by 35%. Pretreatment with Esmo, Inflix 5 mg and Inflix 7 mg significantly increased the level of PGE2 by 188%, 195%, and 230%, respectively, contrasted to the IND group (Table 3).

Table 3: Effect of Inflix on gastric PGE2 levels in indomethacin-treated rats

Groups/Parameters	PGE2 level (pg/ml)
Control group	22.77±0.42
IND group	8.85±0.08**
Esmo Group +IND	17.68±0.34$^{**\#\#}$
Inflix 5mg+IND	16.77±0.95$^{**\#\#}$
Inflix 7mg+IND	18.17±0.50$^{**\#\#}$

$^{**}p \leq 0.005$, significant difference compared with control , $^{\#\#}p \leq 0.005$, significant difference compared to IND, Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab and PGE2: Prostaglandin E2

3.7. Effect of Inflix gastric apoptotic markers in IND-induced GU in rats

Ulcer induction by IND significantly increased BAX and casp-3 by 663% and 530%, respectively, contrasted to the control group, and significantly lowered BCL-2 via 15% with respect to the control group (Fig. 6 A, B, C). Pretreatment with Esmo significantly decreased BAX and casp-3 by 40% and 40%, respectively, compared to IND group, and significantly increased BCL-2 by 546% compared to IND group. Pretreatment with Inflix 5 mg significantly decreased BAX and casp-3 by 35% and 28%, respectively, compared to IND group and significantly increased BCL-2 by 553% contrasted to IND group. Pretreatment with Inflix 7 mg significantly decreased BAX and casp-3 by 24% and 24%, respectively, compared to IND group and significantly elevated BCL-2 by 593% compared to IND group.

3.8. Effect of Inflix on Mitogen-Activated Protein Kinase (MAPK) cascade expression in IND-induced GU in rats

As illustrated in Figure 7, immunoblotting revealed that P-MAPK, P-ERK and P-JNK expression levels were increased in the IND group by 725%, 485%, and 524% concerning the control group, respectively. However, Esmo, Inflix 5 mg and Inflix 7 mg pretreatment decreased P-PMAPK, P-ERK, and P-JNK protein levels by (47%, 42%, and 32%), (47%, 51%, and 30%), and (51%, 49% and 38%) with respect to the IND group, consecutively.

3.9. Effect of Inflix on Nuclear Factor kappa B (NF-κB) cascade expression in IND-induced GU in rats

As illustrated in Figure 8, immunoblotting revealed that P-NF-κB P65 expression levels were increased in the IND group by 713% regarding the control group. However, Esmo, Inflix 5 mg and Inflix 7 mg pretreatment decreased P-NF-κB P65 protein levels via (42%, 38%, and 28%) compared to the IND group.

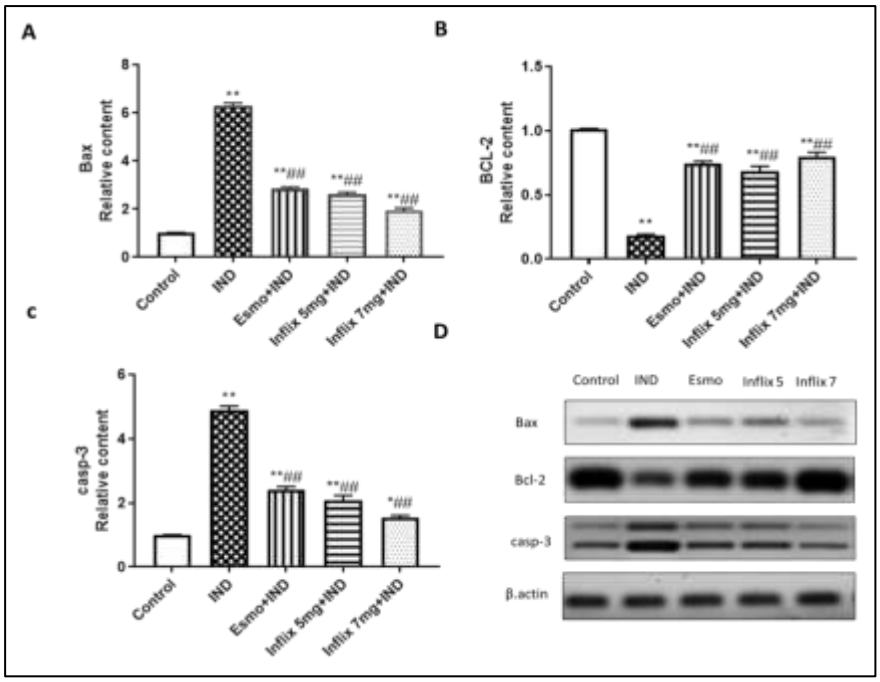

Figure 6: Effect of Inflix on apoptotic factors on IND-induced GU in rats

$^{*}p \leq 0.05$, significant difference compared with control, $^{**}p \leq 0.005$, significant difference compared with control , $^{\#\#}p \leq 0.005$, significant difference compared to IND, Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab, BAX: Bcl-2 associated x, BCL-2: B-cell lymphoma 2 and casp-3: cleaved caspase-3.

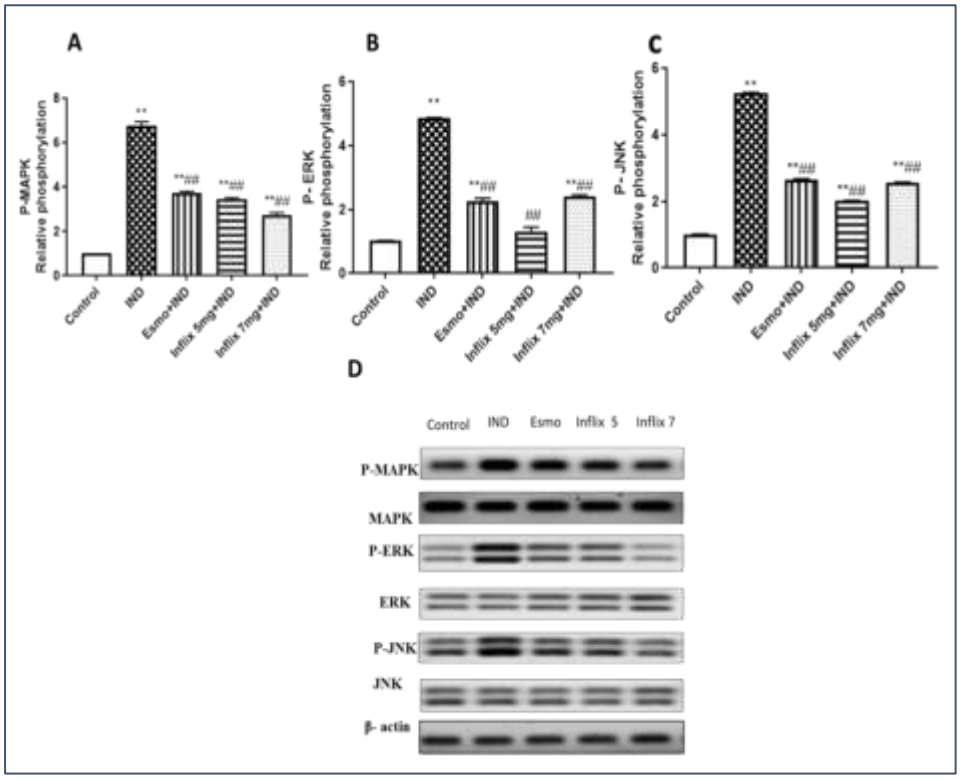

Figure 7: Effect of Inflix on MAPK pathway on IND-induced GU in rats

$^{**}p \leq 0.005$, significant difference compared with control, $^{\#\#}p \leq 0.005$, significant difference compared to IND,Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo group: esomeprazole, Inflix: infliximab, MAPK: mitogen activated protein kinase, ERK: extracellular–signal–regulated kinase and JNK: c-jun N-terminal kinase.

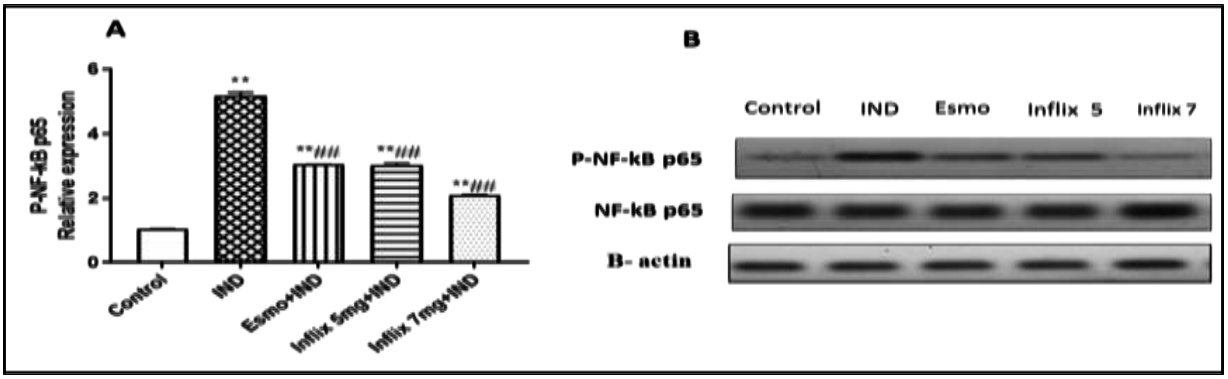

Figure 8: Effect of Inflix on NF-kB pathway on IND-induced GU in rats

$^{**}p \leq 0.005$, significant difference compared with control, $^{\#\#}p \leq 0.005$, significant difference compared to IND, Values mean ± SEM (n=8). Utilizing one-way analysis of variance (ANOVA) accompanied by Turkey-Kramer test for multiple comparisons. Abbreviation: IND: indomethacin, Esmo: esomeprazole, Inflix: infliximab, and NF-kB Nuclear Factor kappa B.

4. DISCUSSION

GU is one of the most prevalent disorders impacting the quality of life [50]. When hostile factors gain the upper hand over protective factors, they lead to ulcers and tissue damage [51].

NSAIDs cause damage to the stomach by inhibiting COX-1, COX-2, and PGE2 (52). According to previous research, the action of IND on PG synthesis is linked to the free radicals' production in ulcerated gastric tissues [53].

Inflix is a monoclonal antibody that works as a free radical scavenger and inflammation inhibitor. Inflix may also prevent TNF-α influenced inflammation via the NF-κB and MAPK signaling cascades [54]. It has proven effective in treating many diseases, such as ulcerative colitis [55].

A macroscopic examination of the stomach was used to assess ulcer formation and the scoring of ulcers. This study supported the hypothesis that IND causes ulcers, proven by the ulcer score. The possible explanation is that gastric damage can easily be produced by forming endogenous and exogenous ROS and free radicals. This finding agrees with previous research [3]. Our findings demonstrated that pretreatment with Esmo decreased the ulcer score contrasted to ulcer-induced rats, which is in harmony with a prior study [20]. The current research aimed to determine how Inflix at 5 and 7 mg doses affected IND-induced GU. Data from the present work indicated that Inflix 5 and 7 mg pretreatment significantly decreased the ulcer score contrasted to IND group. This finding agrees with those of (Freitas et al., 2022), who reported that Inflix decreased the acute inflammatory process in oral ulcers.

In this study, the stomach of the IND group showed the presence of hemorrhagic streaks and deep ulcers. In contrast, Esmo pretreatment showed fewer ulcer numbers compared to IND group, which aligns with a previous result by (El Badawy et al., 2021). The present results showed that pretreatment with Inflix 5 and 7 mg resulted in fewer ulcers as compared to IND group. That agrees with previous research analyzing the Inflix's protective impacts in a rat model of IND-induced enterocolitis [18].

Hematoxylin-eosin stain was used to evaluate the stomach histopathological modifications' extent in rats. The ongoing study observed that ulcer induction by IND caused focal ulceration with hemorrhage and hemosiderosis in gastric mucosal layer, and submucosal edema, and inflammatory cell infiltration, and these outcomes agree with some changes reported in earlier research [58]. In these results, pretreatment with Esmo improved the pathophysiological changes produced by IND, which constitutes the result conducted by researchers [59]. The ongoing work also indicated that all pathophysiological alterations produced by IND were enhanced by pretreatment with Inflix, but to different extents. This result agrees with some changes reported in a previous study, which showed that Inflix pretreatment decreased hemorrhagic areas and inflammatory cell infiltration in lung tissues [36].

It is well defined that the pathogenesis of IND-induced gastric lesions involves the generation of ROS that may have a significant role, namely due to lipid peroxide generation, accompanied by impairment of enzyme activity of the cell [9]. SOD is considered the defense's first line versus the harmful impact of ROS on cells [74]. One of the secondary lipid peroxidation products is MDA, and it can be utilized as a sign of damage to cell membranes [75, 76]. The imbalance between pro- and antioxidant molecules leads to oxidative stress [77]. In the current research, IND significantly decreased SOD and TAC activity and increased MDA levels, leading to oxidative stress. A similar pattern was previously recorded [16, 45]. Pretreatment with Esmo caused a significant elevation in SOD and TAC actions and a decline in MDA levels with regard to IND group. This finding

agrees with prior research [78, 79]. In this study, pretreatment with Inflix 5 and 7 mg demonstrated a significant elevation in SOD concerning IND, which aligns with a study by (Abdelrahman et al., 2018). The present study showed that Inflix 5 and 7 mg pretreatment influenced a significant reduction in MDA content with regard to IND group. This outcome agrees with those who reported that Inflix medication of stressed rats significantly switched the elevation in MDA content [81]. In this study, Inflix 5 and 7 mg pretreatment induced a significant decline in TAC content with regard to the IND group. This result aligns with previous research [80].

Cytokines regulate the mucosal immune defense, including TNF-α, IL-1β, and IL-6, [60-64]. Dysregulation of TNF-α production and other cytokines has been linked with different human diseases, including GU [65]. IL-1β, IL-6, and TNF- α, expressions were significantly raised by IND in this study with regard to the control group. This outcome aligns with prior research [58, 66, 67]. Pretreatment with Esmo significantly reduced TNF-α, IL-1β, and IL-6, level that is similar to previous studies [68, 69]. Our outcomes demonstrated that pretreatment with Inflix demonstrated a significant reduction in TNF-α, IL-1β, and IL-6 level, compared to ulcer-induced rats in a dose dependent manner which agrees with the findings by (Onda et al., 2004; Danese et al., 2006; Poutoglidou et al., 2021; Senousy et al., 2022).

PGE2 can elevate gastric blood flow, stimulate mucus and bicarbonate secretion, hinder acid secretion [90], and even inhibit neutrophil-mediated free radical generation [91, 92]. The ongoing research demonstrated that IND induced a significant decrease in PGE2 regarding the control group, which agrees with previous research [93]. Pretreatment with Esmo significantly increased PGE2 with respect to IND groups [94]. The observed elevation in PGE2 levels in groups pretreated with Inflix is in line with a prior study [47]. We concluded from these studies that Inflix has an anti-inflammatory effect and can easily increase the protective PGE2.

Bax and casp-3, which promote apoptosis when mitochondria are damaged, are crucial in disrupting stomach mucosal integrity. Activating casp-3, the apoptosis' executor, causes programmed cell death. Bcl-2 inhibits Bax's activation and apoptosis [19, 82, 83]. In this study, ulcer induction by IND generated a significant boost in Bax and casp-3 and a reduction in Bcl-2 concerning the control group. Alike pattern was computed previously [21, 84]. Pretreatment with Esmo stimulated a significant reduction in Bax and casp-3 content and an elevation in Bcl-2 content with respect to the IND group, and this result agrees with previous studies by (Ogaly et al., 2021; Taskiran et al., 2021). This study showed that pretreatment with Inflix induced a significant reduction in Bax and casp-3 content and an elevation in Bcl-2 content with regard to the IND group. This result agrees with previous studies [86-89].

The MAPK pathway can be activated by ROS or other inflammatory stimuli, such as ulcer induction by IND or stress. Total MAPK is converted to phosphorylated form then degraded to ERK, JNK, and p38. By causing IκBα to become phosphorylated and separate from the NF-kB p65 subunit, the p38, ERK, and JNK phosphorylation promotes the NF-kB p65 subunit's activation. Increasing the levels of TNF-α, IL-1β, and IL-6 are consequently controlling the several pro-inflammatory genes' expression, which cause tissue and cell inflammatory damage [12, 22, 58, 61, 95-99].

The MAPK cascade was significantly active in the IND group in the current investigation, as predicted, and this finding aligns with other research [58, 100]. Esmo pretreatment lowered the production of the phosphorylated subunits of MAPK, ERK, and JNK. This outcome is consistent with a previous study [101]. Western blotting experiments in the current research demonstrated that the pretreatment with Inflix significantly lowered the high levels of phosphorylation of JNK, MAPK, and ERK in the gastric epithelium stimulated via ulcer induction by IND, suggesting that Inflix could dose-dependently inhibit P-MAPK, P-ERK, and P-JNK signaling pathways in IND-induced GU, which is similar to the finding obtained by cordaro et al. [102].

Although it has long been assumed that IκB breakdown and NF-κB p65 nuclear translocation are necessary for NF-κB activation [28, 103], TNF-α is also the main stimulator for NF-κB expression [104, 105]. Lately, it has been demonstrated that activating the MAPK cascade is essential for activating the NF-κB pathway. The NF-κB pathway was significantly active in the IND group in the current investigation, as predicted by the elevated level of ROS. This outcome is in line with a research that found that IND enhances the NF-κB cascade [59]. Esmo pretreatment lowered the production of the phosphorylated NF-κB p65 subunit. This result agrees with other research [106-108]. The extreme-dose pretreatment improved these impacts with Inflix 7 mg. This outcome is consistent with (Dadsetan et al., 2016; Habib et al., 2019; Younis et al., 2021). The antioxidative effect of Esmo or Inflix may inactivate the MAPK pathway, which weakens the phosphorylation of NF-κB p65, leading to a downregulated expression of TNF-α and IL-1β, simultaneously interfering the positive feedback of TNF-α and IL-1β to the ROS signaling pathways with the production of ROS and the cross-regulation of upstream signaling pathways.

5. CONCLUSIONS

This study shows that besides invasive factors, including stomach acid and pepsin, inflammatory damage produced by ROS is related to a cascade of activations of NF-κB signaling pathways and the MAPK pathway, which are crucial to the pathophysiology of GU. Mucosal damage is aggravated by the positive feedback loop between pro-inflammatory factors and ROS signaling cascades. The anti-inflammatory, anti-oxidative, and apoptotic actions of pretreatment with Esmo or Inflix protect rats from IND-induced GU (fig.9). We also showed that Inflix 7 mg had more significant effects on most of results compared to Esmo or Inflix 5 mg.

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Ethical Statement: The Ethics Committee of the faculty of Pharmacy at Al-Azhar University in Egypt (permission number: 203/2019) was followed in all animal methods. Animal disturbance, pressure, and difficult manoeuvres were avoided.

Author Contribution: All authors contributed to all steps of work.

List of Abbreviations: ANOVA: Analysis of Variance, BAX: Bcl-2 associated X, BCL-2: B-cell lymphoma 2, Casp-3: Caspase-3, CD: Crohn's disease, COX-1: Cyclooxygenase-1, COX-2: Cyclooxygenase-2, ELISA: Enzyme Linked immunosobent assay, ERK: Extracellular –signal – regulated kinase, Esmo: Esomeprazole, GU: Gastric ulcer, IL-1β: Interleukin - 1beta, IL-6: interleukin-6, IND: Indomethacin, Inflix: Infliximab, I.p.: Intraperitoneal, JNK: C- jun-n- terminal kinase, MAPK: Mitogen-activated protein kinase, M arrow: Inflammatory cell infilltration, MDA: Malondialdehyde, Mu: mucosa, Nc: Necrosis, NF-κB p56: Nuclear factor kappa B Nuclear factor kappa B p56, NSAIDs: Non -steroidal anti-inflammatory drugs, O: Oedema, PGE2: Prostaglandin E2, P.o.: per oral, PPIs: Proten pump inhibitors, RA: Rheumatoid arthritis, ROS: Reactive oxygen species, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SEM, Standard error of mean, SOD: Superoxide dismutase, Subm: Submucosa, TAC: Total antioxidant capacity, TBST: Tris buffered saline with tween, TNF-α: Tumor necrosis factor -alpha, Ulc, Haemorrhage.

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