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Mitigation of Indomethacin-Induced Gastric Ulcer in Rats by 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline: Modulation of Inflammatory Mechanisms

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Abstract

Objective: To investigate the gastroprotective influence of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in a rat model of indomethacin-induced gastric ulcers.

Methods: Thirty male Wistar rats were randomly divided into five groups ($n = 6$) as follows: Group 1 (control), Group 2 (indomethacin only, 30 mg/kg), Group 3 (indomethacin and 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, 30 mg/kg), Group 4 (indomethacin with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, 60 mg/kg), and Group 5 (indomethacin with esomeprazole, 30 mg/kg). The efficacy of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in mitigating gastric ulcers induced by indomethacin in rats was evaluated based on gastric morphology, histopathology, and inflammatory biomarkers.

Results: Indomethacin-induced stomach ulcers resulted in epithelial damage and blood streaks on the gastric mucosa. However, treatment with indomethacin and 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline (60 mg/kg) significantly ($P < 0.05$) reduced ulcers compared to the indomethacin-only group. Inflammatory cells were observed in the indomethacin group, while the 60 mg/kg 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline-treated group exhibited the restoration of normal epithelial tissue and minimal inflammatory cells, similar to the control and esomeprazole-treated groups. Furthermore, 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline significantly decreased inflammatory biomarkers (TNF-α, IL-6, INF-γ, and IL-β1) and increased gastroprotective mediator levels (PGE2 and mucin), both with *P*-values below 0.05, in contrast to the effects of indomethacin.

Conclusion: This study provides clinical evidence highlighting the gastroprotective properties of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline. Its initial application reveals, for the first time, its efficacy in treating gastric ulcers induced by indomethacin. However, further tests are warranted to validate these findings.

Keywords: Gastric ulcer, 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, indomethacin, inflammatory biomarkers, esomeprazole, ELISA

Introduction

Gastric ulcers, impacting 10% of the global population, pose significant threats to the gastrointestinal tract, leading to life-threatening implications and mortalities. Controlling and detecting them early present major challenges.[1-3] The emergence of gastric ulcers stems from an imbalance between the factors that affect the stomach's health and those that provide protective mechanisms. This delicate equilibrium between destructive and defensive elements contributes to the development of gastric ulcers.[4] Prominent aggressive factors encompass heightened gastric acid secretion, ethanol intake, irregular motility, smoking, *Helicobacter pylori* infection, and the usage of non-steroidal anti-inflammatory drugs (NSAIDs).[5] These elements compromise the integrity of the gastric mucosa, resulting in the deposition of intracellular calcium.[6]

Conversely, essential protective elements involve prostaglandin synthesis, mucus secretion, generation of bicarbonate, and maintaining a regular blood supply to the tissue.[5] In the Kingdom of Saudi Arabia, there is a prevalent and substantial daily consumption of NSAIDs.[7] *Helicobacter pylori*, a clinically significant pathogen, establishes colonization in approximately 50% of the global population. Additionally, *Helicobacter pylori* infection is markedly present within the Saudi population suffering from gastric ulcers.[8]

The imbalance between damaging factors and protective mechanisms in the gastrointestinal mucosa can lead to gastric irritation and ulceration. Prolonged anxiety, stress, surgical shock, burns, and trauma all contribute to this imbalance. Oxygen-derived free radicals are known to be implicated in the pathogenesis of gastric damage caused by various factors in humans and animals.[4]

NSAIDs stand out as widely employed medications for inflammatory conditions, offering efficacy in alleviating pain, managing fever, and addressing redness and edema caused by the release of inflammatory mediators.[9] Indomethacin is the

preferred choice for creating an experimental ulcer model due to its heightened ulcerogenic potential compared to other NSAIDs. It works by suppressing prostaglandin synthesis through the inhibition of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes. The anti-inflammatory effect of indomethacin is attributed to COX-2 enzyme inhibition, while the inhibition of COX-1 enzyme is responsible for its gastrointestinal side effects. Literature reports indicate that indomethacin induces gastric damage by hindering the production of COX-1 enzyme-derived prostaglandin E-2 (PGE-2), bicarbonate, and mucus. Additionally, it promotes gastric acid secretion, elevates oxidant parameters, and diminishes antioxidant parameters.[6,9-11]

Common synthetic antiulcer medications such as cimetidine, misoprostol, ranitidine, and omeprazole are utilized for treating NSAID-induced gastric ulcers. Importantly, each of these drugs is associated with a spectrum of side effects, ranging from mild to severe.[12,13] Quinoxaline compounds have diverse applications, demonstrating varied biological properties which are useful in cancer therapy and antimicrobial development.[14] The natural presence of 2,3-dimethylquinoxaline (DMQ) in the *Chromolaena odorata* plant has been documented.[15] The quinoxaline structure allows a number of activities, acting as a precursor for the synthesis of numerous compounds with various applications.[16] We propose that 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline has gastroprotective effects against indomethacin-induced gastric ulcers through the application of an animal experimentation model.

Materials and Methods

Drugs and Chemicals

The compounds, namely 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, indomethacin, esomeprazole, and carboxymethyl cellulose sodium (CMC-Na), were acquired from Sigma-Aldrich in St. Louis, MO, USA. The experimental setup also incorporated several ELISA kits, including the Rat TNF-α ELISA Kit, Rat Interferon Alpha kit (Cat No. MBS267050), Rat Prostaglandin E2 (PGE2) ELISA Kit (Cat No. MBS262150), Rat Mucin ELISA (Cat No. MBS1600651), Rat Interleukin 6 (IL-6) ELISA Kit (Cat No. MBS269892), Rat Inducible Nitric Oxide Synthase (iNOS) Elisa kit (Cat No. MBS723326), ELISA kit (Cat No. MBS725633), and Rat IL-1 beta ELISA Kit (Cat No. MBS825017), all obtained from Sigma-Aldrich in St. Louis, MO, USA. Various commercially available chemicals, such as formalin, phosphate buffer, and other essential compounds, were selected in elevated purity grades. It is paramount to emphasize that every chemical utilized throughout the study adhered to the standards of analytical grade.

Esomeprazole, widely utilized as a protective agent against gastric ulceration, served as a reference drug in the study. This was in accordance with various research studies on gastroprotective actions employing esomeprazole as a reference drug.[17]

Study Design and Animal Handling

The animal-related procedures in this study strictly adhered to the approved protocols set by the Research Ethics Committee of the Faculty of Pharmacy at King Abdulaziz University, KSA (Reference No "PH-1444-55"). Male Wistar rats, aged 10 weeks and weighing between 200 and 230 grams, were obtained from the Faculty of Pharmacy's animal facility at King Abdulaziz University. These rats were housed in a controlled environment, with a temperature maintained between 20–24°C and a 12-hour light and 12-hour dark cycle. They were provided with unlimited access to a standard diet and water, and a one-week acclimation period was implemented before the commencement of experiments.

A total of thirty rats were randomly assigned to five groups, each comprising six rats, and subjected to the following treatments:

1. Group 1 (negative control): Rats orally received the vehicle (0.5% w/v carboxymethyl cellulose sodium, 10 mL/kg).
2. Group 2 (positive control): Rats in this group were administered a single oral dose of indomethacin (30 mg/kg).
3. Group 3: indomethacin + 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline 30 mg/kg: Rats in this group were given 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline orally at a dose of 30 mg/kg for three consecutive days. On the third day, they received indomethacin (30 mg/kg) orally, followed by the last dose of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline one hour later.
4. Group 4: indomethacin + 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline 60 mg/kg: Rats in this group received 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline at a dose of 60 mg/kg orally for three consecutive days.
5. Group 5: indomethacin + esomeprazole Group: Rats in this group were orally administered esomeprazole (30 mg/kg) for three consecutive days. On the third day, they received indomethacin (30 mg/kg) orally, followed by the last dose of esomeprazole one hour later.

After 4 hours of indomethacin administration, rats in the treatment groups were euthanized for subsequent analysis.[18]

Induction of Gastric Ulcers

Aligned with earlier investigations, the induction of gastric ulcers was observed following the administration of indomethacin. Fasting for 24 hours was implemented on the second day of the experiment, allowing access only to water. On the third day, all groups, except the control group, were subjected to intragastric administration of indomethacin at a dose of 30 mg/kg, suspended in a 0.5% carboxymethyl cellulose sodium (CMC-Na) solution.[19]

Analysis of Morphology and Histopathology

After the dissection of the animals' stomachs, they underwent a thorough rinse with normal saline (0.9% NaCl) and were digitally photographed. Subsequently, the stomachs were examined under a microscope to identify any hemorrhagic lesions within the glandular mucus layers. Following the outlined procedure, the stomach samples were preserved in a solution containing 10% formalin and saline.

Then the gastric tissues underwent a series of steps, including washing, dehydration with progressively increasing concentrations of alcohol, clarification with xylene, and eventual embedding in paraffin wax. Thin sections, approximately 5 µm thick, were prepared and subjected to staining with hematoxylin and eosin (H & E) to reveal structural alterations. A thorough tissue examination was conducted using a light microscope.

To evaluate histopathological changes, a scoring system ranging from 0 to 4 was implemented in the study. This scoring system, applied by a histopathologist blinded to the

specific treatments, took into account factors such as edema in the gastric mucosa, infiltration of inflammatory cells, gastric hemorrhage, and necrosis.[18]

Inflammatory Biomarker Assessment

In the evaluation of inflammatory biomarkers, gastric tissue homogenates underwent thorough analysis to determine PGE2 activity utilizing the Rat Prostaglandin E2 (PGE2) ELISA Kit (Cat No. MBS262150, St. Louis, MO, USA). Furthermore, the assessment of IL-6, TNF-α, IFN gamma, and Rat IL-1 beta in the supernatant was carried out employing their respective ELISA Kits (Cat No. MBS269892, MBS2507393, MBS267050, MBS825017). Measurement of mucin protein was conducted using the Rat MUC1 ELISA kit (Cat. No. MBS1600651, St. Louis, MO, USA). All procedures strictly adhered to the manufacturers' protocols, and the kits utilized were sourced from Sigma-Aldrich in St. Louis, MO, USA.[18]

Statistical Analysis

The data presented in this study are expressed as the mean ± standard deviation (SD). Multiple comparisons were conducted through one-way ANOVA, followed by Tukey's post-hoc test for further analysis. A probability value (*P*) less than 0.05 was established as the threshold for statistical significance. All statistical analyses were performed using GraphPad Instat software version 3. Graphs were generated using GraphPad Prism software version 8 (GraphPad Software, Inc., USA).

Results

Impact of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on Gastric Morphology

The effectiveness of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in mitigating indomethacin-induced peptic ulcer damage was assessed using rat stomach mucosa. In Figure 1A, the stomach from the control group (Group 1) displayed normal mucosa without any injuries. Conversely, the indomethacin group (Group 2) exhibited bloody streaking wounds (Figure 1B). Notably, the indomethacin + 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline (30 mg/kg) group (Group 3) demonstrated a reduced occurrence of bloody streaking (Figure 1C). Rats treated with indomethacin and 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline (60 mg/kg) (Group 4) exhibited a significant decrease in the ulcer index compared to the indomethacin group, accompanied by evidence of mild injuries (Figure 1D). As expected, esomeprazole treatment (Group 5) led to a noteworthy reduction in the ulcer index compared to the group receiving only indomethacin, effectively preserving the integrity of the gastric mucosa layer (Figure 1E). These findings suggest that 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline demonstrates comparable efficacy to esomeprazole.

Impact of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on the Histopathological Features of Rat

Histological analysis was employed to assess the impact of indomethacin, 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, and esomeprazole on rat stomach mucosa. In the normal controlled group (Figure 2A), the epithelial tissue exhibited the typical histological structure of the gastric tissue, devoid of inflammatory cells. Figure 2B exhibited inflammatory cells, the rats exposed to indomethacin displayed damaged lining epithelium, and focal ulceration (Figure 2B). Histopathological examinations exhibited less severe damage to the lining epithelium of the mucosal layer, along with a reduction in the total number of inflammatory cells and hemorrhage in the 30 mg/kg treatment group (Group 3) compared to the indomethacin-treated group (Group 2) (Figure 2C). However, the 60 mg/kg 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline group (Group 4) displayed the restoration of normal epithelial tissue and minimal inflammatory cells, resembling the control

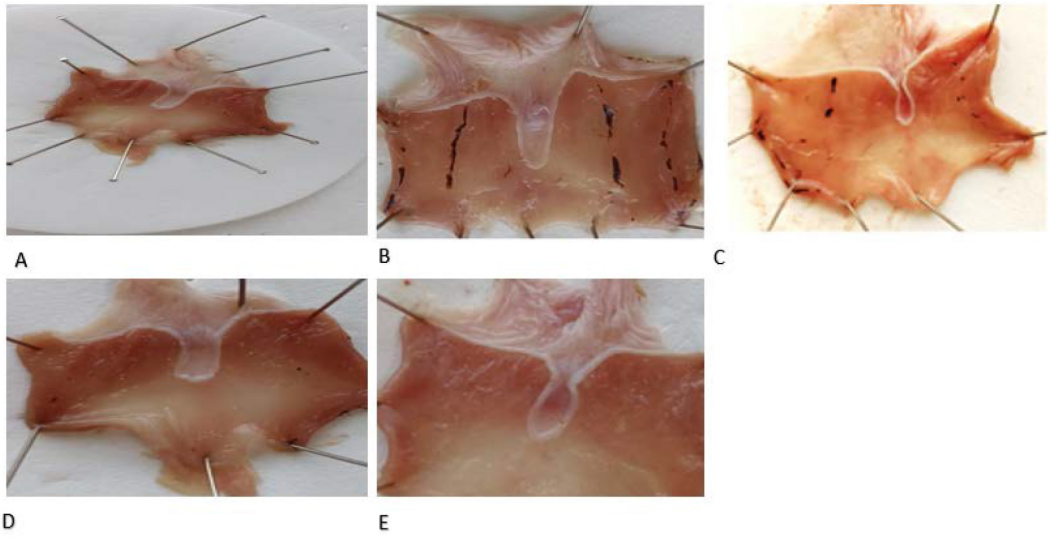

Fig. 1 **Photographs of rat stomachs' macroscopic appearance: (1A) The control group exhibited a healthy stomach mucosa with no lesions or redness. (1B) Rats treated with indomethacin displayed severe bleeding and mucous surface ulceration. (1C) indomethacin + 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline (30 mg/kg) showed surface injuries. (1D) indomethacin + 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline (60 mg/kg) showed minimal damage with a healthy mucosa. (1E) indomethacin + esomeprazole (30 mg/kg) efficiently restored the injured mucosal layer to normal, displaying no visible redness or damage.**

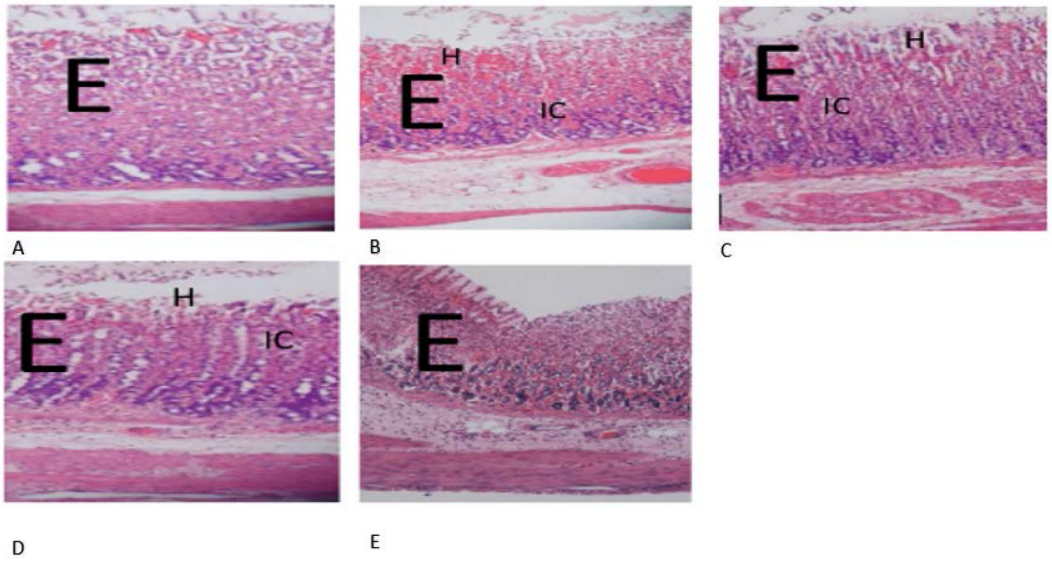

Fig. 2 **Depicts images from the histopathological examination of the epithelial tissue. A represents the negative control, B is the ulcer control (received only indomethacin), C is the group treated with a low dose of 30 mg/kg 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, D depicts high doses of 60 mg/kg 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline, and E represents the group that received esomeprazole 30 mg/kg. It is evident that with different doses of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in C and D, or esomeprazole 30 mg/kg in E, there is a restoration of the normal surface epithelial layer (labeled E) with less hemorrhage (labeled H) and fewer inflammatory cells (labeled IC) compared to B.**

group (Group 1) and the group treated with esomeprazole (Figure 2D, 2E). This suggests that 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline can function as an anti-ulcer agent for the stomach, with higher doses corresponding to greater improvements in rat stomach histology.

Influence of Pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on Inflammatory Markers

In the rat ulcer model, the effectiveness of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline was assessed through molecular markers, such as TNF-α, IL-6, IL-1β, IFN-gamma, and INOs. Illustrated in Figure 3, exposure to indomethacin initiated a notable pro-inflammatory response, evidenced by a substantial increase in TNF-α, IL-6, IL-1β, IFN-gamma, and INOs concentrations in gastric tissues, in contrast to the control group that did not receive indomethacin. However, pre-treatment with esomeprazole (30 mg/kg) or 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline at doses (30 and 60 mg/kg) exhibited an anti-inflammatory effect, significantly ($P < 0.0001$) reducing TNF-α, IL-6, IL-1β, IFN-gamma, and INOs concentrations compared to the indomethacin-treated group. These findings suggest that the effectiveness of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in modulating molecular mediators in inflammatory processes is comparable to that of esomeprazole (Figure 3).

Influences of Pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on Concentrations of PGE2 and Mucin

Illustrated in Figure 4, the results unveiled a notable decrease in mucin and prostaglandin levels following indomethacin administration in comparison to the negative control (untreated group). Conversely, the group pretreated with esomeprazole exhibited a substantial ($P < 0.0001$) elevation in PGE2 and mucin concentrations relative to the indomethacin-exposed group. Furthermore, 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline pre-treatment at doses of 30 and 60 mg/kg demonstrated noteworthy ($P < 0.0001$) increases in PGE2 and mucin concentrations in a dose-dependent manner when compared to the indomethacin-exposed group. This indicates similar efficacy of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline and esomeprazole in enhancing the concentration of both PGE2 and mucin.

Discussion

NSAIDs contribute to a 15% elevation in the occurrence of gastric and peptic ulcers during endoscopic examinations Indomethacin,[20] among NSAIDs, is recognized for its substantial ulcerogenic potential and is commonly employed to induce experimental ulcer models in animals.[6] Existing treatments for gastric ulcers exhibit both adverse reactions and limited effectiveness against gastric disorders.[21] Consequently, there is a current focus on medicinal research to develop plant-origin anti-ulcer drugs that are safe and efficacious. To our knowledge, this study represents an inaugural analysis of the anti-ulcerative properties of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline in a rat model of indomethacin-induced ulcers.

The NSAID-induced ulcer model, renowned for its popularity, is based on the disruption of the equilibrium between anti-inflammatory and pro-inflammatory mediators at the injury site. This imbalance involves reduced prostaglandin E2 and heightened secretion of IL-6 and TNF-α by epithelial cells.[11,22,23] Hence, the NSAIDs, particularly the indomethacin-induced ulcer model is employed for the purpose of the present study.

In the present investigation, the administration of indomethacin-induced substantial morphological damage, histopathological alterations, and a decrease in mucin content

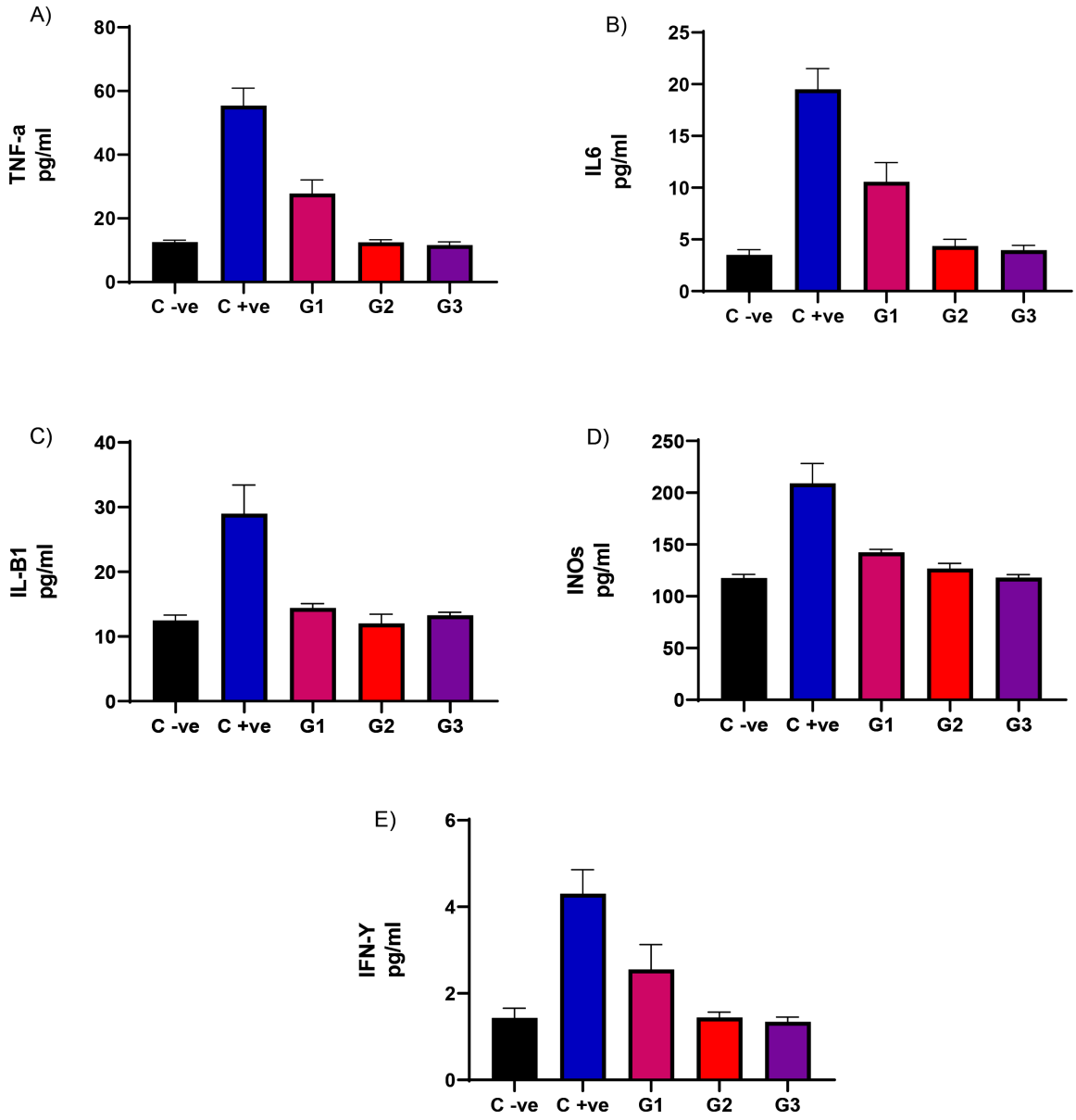

Fig. 3 **Shows the influence of pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on various molecular markers, including A) TNF-α, B) IL-6, C) IL-1β, D) INOs, and E) IFN-gamma concentrations in rats exhibiting indomethacin-induced gastric ulcers. The data, represented as mean ± S.D. ($n = 6$), exhibited statistical significance compared to both the corresponding control and indomethacin groups at $P < 0.05$. This determination was made using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.**

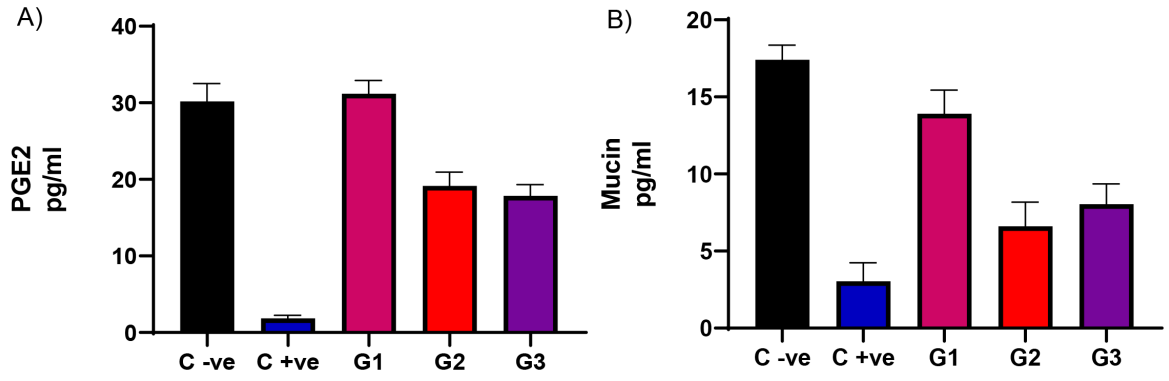

Fig. 4 **The impact of pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline on concentrations of A) PGE2 and B) Mucin in rats experiencing indomethacin-induced gastric ulcers is illustrated. The data, expressed as mean ± S.D. ($n = 6$), exhibited statistical significance compared to both the corresponding control and indomethacin groups at $P < 0.05$. This statistical determination was conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.**

in the gastric tissues of rat stomachs. Conversely, pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline at doses of 15 and 30 mg/kg exhibited mitigated injuries compared to the indomethacin-exposed group. The highest 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline dose demonstrated minimal injuries with no observable histopathological changes. Notably, 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline exhibited an enhanced of mucin at all tested doses. Our results align with prior research, demonstrating that pre-treatment with tetramethylpyrazine led to an elevation in mucin concentration and a reduction in the levels of TNF-α and IL-6. Furthermore, their findings indicated that the tetramethylpyrazine-treated group exhibited improvements in both morphological and histopathological changes when compared to the peptic ulcer group induced by indomethacin.[18]

Gastric ulceration arises from an augmented concentration of reactive oxygen species, encompassing hydrogen peroxide, hydroxyl radicals, and superoxide anions. The ensuing oxidative stress within the gastric tissue plays a pivotal role in the occurrence of gastric bleeding and the subsequent development of ulcers.[24] Moreover, the oxidative stress induced by indomethacin contributes to mitochondrial respiration uncoupling, leading to inflammation and the production of pro-inflammatory cytokines, including TNF-α and IL-6. These cytokines, in turn, instigate the upregulation of adhesion molecules, such as ICAM-1, which play a pivotal role in the initiation and progression of injury and inflammation within the gastric tissue.[18,23] In the present investigation, pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline exhibited a noteworthy reduction in the concentrations of inducible nitric oxide synthase INOs, TNF-α, IL-1, and IL-6 when compared to rats exposed to indomethacin. This led to a consequential decrease in inflammation. These outcomes align with a prior study where tetramethylpyrazine demonstrated a capacity to mitigate the production of inflammatory cytokines.[18] The findings from the ongoing investigation underscore the notion that gastric injuries arising as an adverse outcome of NSAID usage primarily stem from the inhibition of cyclooxygenase (COX) enzymes. This inhibition, in turn, hampers the synthesis of prostaglandin E2 (PGE2), resulting in compromised or diminished gastric protection. Notably, PGE2 adopts a pivotal role in fostering mucus production and enhancing gastric blood flow, thereby orchestrating a gastro-protective cascade.[25] In the comprehensive scope of our study, a discernible reduction in gastric prostaglandin E2 (PGE2) and mucin levels was unequivocally demonstrated upon indomethacin exposure. However, it is noteworthy that pre-treatment with 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline exerted partial mitigation of this effect, showcasing its potential role in ameliorating the adverse impacts induced by indomethacin on gastric PGE2 and mucin concentrations. According to existing literature, prostaglandin E2 (PGE2) is recognized for its healing-promoting attributes, notably linked to angiogenesis induction by stimulating vascular endothelial growth factor (VEGF) production in fibroblasts, thereby fostering cell proliferation. Furthermore, a recent investigation involving rats corroborated these findings, revealing an augmentation in gastric mucin content, alleviated neutrophil infiltration (as indicated by reduced myeloperoxidase activity), and a decline in elevated serum nitric oxide levels.[26,27]

In summary, our study marks the initial demonstration of the protective efficacy of 2,3-Dichloro-6-(trifluoromethoxy) quinoxaline against indomethacin-induced gastric ulcer in rats. This observed protection is attributed, at least in part, to its antioxidant and anti-inflammatory properties. However, it is imperative to note that additional comprehensive investigations are warranted to delve deeper into the underlying mechanisms and substantiate these preliminary findings.

Statement of Potential Conflicts of Interest

The authors of this research paper affirm unequivocally that they do not possess any identifiable competing financial interests or personal affiliations that might be perceived to exert influence over the outcomes presented in this manuscript. This transparent disclosure is provided to maintain the integrity and credibility of the scientific work detailed herein.

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