RESEARCH ARTICLE

The Potential Protective Effects of Pirfenidone on Diclofenac Sodium Induced Gastric Ulcer in a Rat Model

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ABSTRACT

Introduction: Non-steroidal anti-inflammatory drugs (NSAIDs), are widely prescribed in clinical practice because of their ability to reduce pain, fever, and inflammation. However, NSAID-induced gastric mucosal damage is the major side effect of these medications. This study aims to reduce gastric mucosal lesions caused by NSAIDs by using pirfenidone by investigating their effect on histological, gastric gross mucosal damage.

Method: 30 healthy male albino rats were divided into 3 groups each of ten (N=10). A single oral dose of diclofenac sodium (150 mg/kg body weight) was used to induce ulceration for all groups except first. The vehicle (tween 80+NS) was given to the first group, while diclofenac sodium was given to the second group and to all pre-treated groups. The third group were pre-treated with pirfenidone (300 mg/kg). At the end of the experiment, histological examination, and antioxidant and anti-inflammatory parameters by immunohistochemistry method were evaluated**.**

Results: Diclofenac sodium at a dose (150 mg/kg) produces a significant increment ($p > 0.01$) in gastric damage score, the expression of tumor necrosis factor-alpha (TNF-α), myeloperoxidase, malonaldehyde, and the ulcer formation percent, compared to the healthy rat's group. Pirfenidone at a dose of (300 mg/kg) pretreatment in diclofenac induced-ulcer in rats produces a significant reduction ($p > 0.01$) in gastric damage score, the expression of TNF-alpha, myeloperoxidase, the expression of TNF-alpha, myeloperoxidase, malonaldehyde, and in the ulcer formation percent, yet, less effectively than omeprazole and pirfenidone.

Conclusion: Diclofenac can be reduced or prevented by the pretreatment of pirfenidone. Pirfenidone showed similar results to the standard treatment (Omeprazole), the protective effect of pirfenidone was mainly through their antioxidant and anti-inflammatory activity by reducing oxidation markers like MPO and MDA, and also reducing inflammatory cytokines like TNF-alpha.

Keywords: Diclofenac sodium, Gastric ulcer, Protective effects, Pirfenidone, Rat model.

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INTRODUCTION

Abrasion of gastric lining epithelium occurs due to an imbalance between gastric defense, protective mechanism and aggressive factors represent about 20% of peptic ulcer start as mild erosion of epithelia lining of the stomach lumen and extend deeper to *muscularis* mucosa or submucosa in 5 mm in diameter or greater.[1] Gastric ulcer (GU) is the most widespread gastrointestinal tract disease; approximately 5 to 10% of populations are affected.[2] A multifactorial GU disease leads to prevalence differences between countries and appears to be more prevalent in developing countries and densely populated regions than in developed countries due to low socioeconomics and hygiene habits.[3] GU is highly linked to *Helicobacter pylori* infection and chronic non-steroidal anti-inflammatory drugs (NSAID) use, the incidence of GU is about 80% in *H. pylori* infected than non- infected patients and 10 to 30% of gastric ulcer is related to chronic NSAID use.[4] *H. pylori* prevalence increases with age (53.3%) and appear to be more in female 59.72% than male 43.75%.[5] GU is a multifactorial disease, endogenous and exogenous factors involved in GU development. GU is highly related to *H. pylori* infection a common human pathogen responsible for ulcer formation usually found beneath the mucus layer, multi virulent factors involved in *H. pylori* toxic effect.[6] NSAIDs play an important role in GU formation by reducing gastric defense layers through inhibition of prostaglandin synthesis by blocking cyclo-oxygenase (COX) enzyme isoforms.[7] Parietal cells secret acid and considered the first line of defense mechanism against bacterial overgrowth and colonization also enhance the absorption of some important materials, including iron, calcium and B12. Overproduction of gastric acid cause mucosal damage by entering the gastric lumen through channels in

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mucus layer created by high glandular hydrostatic pressure during secretion. Thus, lead to convert superficial erosion to a deeper lesion, disturb mucosal integrity and inactivates acid liable factors.[8] Analgesic, anti-inflammatory and antipyretic are weak acid drugs with good gastrointestinal absorption, high protein binding and bioavailability. It's most widely used worldwide, especially by the elderly to relieve rheumatoid arthritis pain.[9] Pirfenidone is a vel anti fibrotic agent with minimal adverse effects.[10] Pirfenidone has been proved for the treatment of idiopathic pulmonary fibrosis disease (IPF).[11] Pirfenidone has a relatively good safety profile and is generally well tolerated. Most common side effects are gastrointestinal (diarrhea, nausea and dyspepsia) plus photosensitivity (skin rashes), as in for laboratory abnormalities it includes an elevation in the level of transaminases.[12] This study aims to reduce gastric mucosal lesions caused by NSAIDs by using pirfenidone and investigating their effect on histological, gastric gross mucosal damage.

MATERIALS AND METHODS

The study was a prospective, comparative, pre-clinical study. After obtaining ethical approval from collage of medicine university of Baghdad, the study was conducted at the Iraqi center of cancer research and medical genetics, Baghdad, Iraq and lasted (5) months from March, 2022 till August and 2022. This study was conducted on 30 adult males albino wistar rats weighing (250–280 g), purchased from the Iraqi center of cancer research and medical genetics, Baghdad-Iraq. Animal were housed five per cage for one week prior to the experiment, they were maintained in normal laboratory conditions (25 ± 2°C, 12 hours light-dark cycle) and had access to laboratory chow pellet with full access to tap water. The animals were divided in to five groups, ten rats in each group and as following: Group A: the animals did not receive any ulcerogen or test material except with the vehicle (0.9% NaCl and tween 80) *via* oral gavage rout, which was used to prepare the test material and this group served as negative control group. Group B: the animals received the ulcerogen (diclofenac sodium 150 mg/kg) *via* oral gavage rout and were no pre-treated with any reference drug or test material and this group will serve as positive control group. Group C: the animals received with the ulcerogen (diclofenac sodium) and prior to the ulcerogen the animals received with pirfenidone 300 mg/kg *via* oral gavage rout. As the prior feeding of the animal has been shown to minimize the ulcerogenic action of some drugs, the animals were fasted for 24 hours before the administration of diclofenac sodium, during the fasted period the rats had full access to tap water which was held two hours before the procedure. All drugs were freshly prepared before administration on the day of the experiment. Diclofenac sodium was used for the induction of GU at a dose of (150 mg/kg) body weight in water, orally at a concentration (20 mg/mL) dissolved in normal saline (NaCl 0.9%) to which tween 80 was added (2 drops of tween 80) is a widely used non-ionic emulsifier that is added to cosmetics, pharmaceuticals, and foods (Nazar, M. *et al.,* 2021) in 10 mL 0.9% NaCl), tween 80 was used as surfactant and wetting agent. Pirfenidone, and omeprazole were used as a protectant against gastric mucosal damage induced by diclofenac sodium, they were dissolved in the vehicle (0.9% NaCl and tween 80) at doses of 200, 300 and 20 mg/kg, respectively. Tissue of rats of all groups was harvested at the end of the experiment and histopathological changes of stomach of each rat were evaluated and scored as follows.[11] Quantification of protein expression was evaluated under light microscopy at 20X. The extent of the immunohistochemical reaction of proteins, such as MDA, MPO and TNFα was measured by percentage of positively stained cells according to the following scale.[12] The software used for data analysis was SPSS (Statistical Packages for Social Sciences) version 26. The data were represented by their mean, standard deviations, and 95% confidence level in graphs. The analysis of variances (ANOVA) test was used to compare the variable mean according to study groups, then least the square differences test was used as a post- hoc test to find the comparable differences among groups.

RESULTS

Intra-gastric administration of (150 mg/kg) diclofenac sodium on an empty stomach, caused extensive multiple hemorrhagic lesions (100% induction). Diclofenac sodium caused significant ($p < 0.01$) mucosal injury represented as gastric damage score (the total linear lengths of lesions represent ulcers number and severity, the severity). The ulcer's number score mean of diclofenac sodium was found to be (3.8 ± 0.4) with (100%) ulcer formation, while the mean scores of lesion severity were found to be (4 ± 0) with (100%) ulcer formation versus (0.0) for a healthy group, Table 1. Intra-gastric administration of (300 mg/kg) pirfenidone 1-hour prior to diclofenac sodium administration significantly reduced diclofenac sodium-induced gastric mucosal injury. Pirfenidone caused a significant reduction ($p < 0.01$) in gastric damage score mean, the ulcer's number score mean of pirfenidone was (1.1 ± 0.3) with (28.9 ± 8.3%) ulcer formation versus (3.8 ± 0.4) with (100%) ulcer formation for diclofenac sodium, while the mean scores of lesion severity for pirfenidone were found to be (1.2 ± 0.4) with (30 ± 10.6%) ulcer formation versus (4 ± 0) with (100%) ulcer formation for diclofenac sodium as show in Table 1.[13]

Microscopic investigation of this group showed deep ulceration, severely eroded epithelium, and disoriented villi and crypts. Mucosal and sub-mucosal congestion were also noted. A dense inflammatory infiltrates in the lamina propria and hemorrhagic patches were observed in severely ulcerated sections after intra-gastric administration of (150 mg/kg) diclofenac sodium on empty stomach. The mean microscopic damage score in the diclofenac sodium group was found to be (4 ± 0) with a (100 ± 0%) chance of ulcer formation, which was significantly higher than the healthy group and all other groups, Table 2. Microscopic investigation of this group showed a very close to normal view, with some epithelial cells erosion, normal parietal cell distribution, normal gastric pit, and normal surface epithelium with a few detached superficial epithelial cells. The mean microscopic damage score in the pirfenidone group was (1 ± 0) with (1 ± 0) chance of ulcer formation which was

Table 1: Effect on gastric lesions number score, severity and damage percentage of indomethacin ulcerated rats, according to study groups

Groups	*Mean ± SD*	*Damage %*	*Severity score*	*Damage %*
Healthy	0 ± 0 A	0 ± 0% A	0 ± 0 A	0 ± 0% A
Diclofenac sodium	3.8 ± 0.4 B	100 ± 11.1% B	4 ± 0 B	100 ± 0% B
Pirfenidone + Diclofenac	1.1 ± 0.3 D	28.9 ± 8.3% D	1.2 ± 0.4 C	30 ± 10.6% C
P-value	<0.001**	<0.001**	<0.001**	<0.001**

ANOVA test, **significant at 0.01

Table 2: Effect on mean histopathological damages scores comparison, according to study groups.

Groups	*Histopathology damage scores*	*Damage %*
Healthy	0 ± 0 A	0 ± 0% A
Diclofenac sodium	4 ± 0 B	100 ± 0% B
Pirfenidone + Diclofenac	1 ± 0 E	25 ± 0% E
p-value	<0.001**	<0.001**

ANOVA test, **significant at 0.01

significantly lower than the diclofenac sodium group which was (4 ± 0) with a (100 ± 0%) chance of ulcer formation, Table 2.

Diclofenac sodium shows a very high expression of MPO. The mean MPO score in this group was (4 ± 0) with a damage score% of (100 ± 0%), showing significant differences from other groups, this means the lowest expression of this protein is found in pirfenidone and finally diclofenac sodium, the latter shows the highest expression of the protein as show in Table 3.

Diclofenac sodium bears the highest expression of the protein (TNF-alpha) with mean TNF scores for this medication (4 ± 0) and damage score% of (100 ± 0%), showing significant differences from other groups, this means the lowest expression of this protein is found in pirfenidone, and finally diclofenac sodium, the latter shows the highest expression of the protein as show in Table 4. Diclofenac sodium with a very high expression of MDA, the mean MDA score in this group was (3.8 ± 0.4) with a damage score% of (100 ± 11.1%), showing significant differences from other groups, this means a decrease in the expression of this protein in pirfenidone treated group compared to the diclofenac sodium as show in Table 5.

DISCUSSION

GU is one of the most common chronic gastrointestinal diseases characterized by a significant defect in the mucosal barrier. The frequent long-term use of NSAIDs and *H. pylori* infection are major factors involved in gastric ulcer development. Acid inhibitors and antibiotics are commonly used to treat the GU. However, in the last few decades, the accumulating evidence for resistance to antibiotics and the side effects of antibiotics and acid inhibitors have drawn attention

Table 3: Mean MPO scores comparison, according to study groups.

Groups	*MPO score*	*Damage%*
Healthy	1 ± 0 A	27 ± 0% A
Diclofenac sodium	3.7 ± 0.5 B	100 ± 13.1% B
Pirfenidone + Diclofenac	1.2 ± 0.4 A	32.4 ± 11.4% A
p-value	<0.001**	<0.001**

ANOVA test, **significant at 0.01.

Table 4: Mean TNF scores comparison, according to study groups

Groups	*TNF score*	*Damage%*
Healthy	1 ± 0 A	25 ± 0% A
Diclofenac sodium	4 ± 0 B	100 ± 0% B
Pirfenidone + Diclofenac	1.2 ± 0.4 A	30 ± 10.6% A
p-value	<0.001**	<0.001**

ANOVA test, **significant at 0.01

Table 5: Mean MDA scores comparison, according to study groups

Groups	*MDA score*	*Damage %*
Healthy	1 ± 0 A	26.3 ± 0% A
Diclofenac sodium	3.8 ± 0.4 B	100 ± 11.1% B
Pirfenidone + Diclofenac	1.3 ± 0.5 A	34.2 ± 12.7% A
p-value	<0.001**	<0.001**

ANOVA test, **significant at 0.01

to the possible use of new medications in preventing and treating GU.[14] Diclofenac, one of the most effective NSAIDs, has several adverse effects on the gastrointestinal tract by the overproduction of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-8) is considered to be an important inducer of these lesions.[15] Because of its inhibitory effect on prostaglandin synthesis, it can harm stomach tissue by increasing gastric acid and pepsin activity and lipid peroxidation and oxidative stress by generating free radicals like (MDA, and MPO).[16] In this study the sites where diclofenac sodium-induced gastric mucosal lesions were the glandular portion of the rat's stomach. The macroscopic appearance of these mucosal lesions is in accordance with the finding, in which they expressed a very similar gastric mucosal damage with close features for this current study.[17,18] The present study revealed that there is a significant increase in the lesion number and lesion severity (which is represented by the total linear lengths of lesions). in ulcerated rats following diclofenac sodium oral administration (150 mg/kg). These findings agree with the findings of (Mostafa, R.E. *et al.,* 2020); and (Tandoh, A. *et al.,* 2021), these studies show that diclofenac does increases lesions number and severity significantly due to the mechanisms mentioned above.[17,18] The increased levels of MPO and MDA in the stomach of diclofenac-ulcerated rats is a symptom of accelerated lipid peroxidation and free radical overproduction, resulting in mucosal injury, according to the current study which came in agree with (Akinrinde, A.S. and Hameed, H.O., 2022).[19] In which they expressed an elevation of serum MPO activity (83.30%), when compared with control, and also in agreement with (Fornai, M. *et al.,* 2020)[20] in which they expressed increments of MPO and MDA, overexpression. One of these drugs is pirfenidone, not a large number of studies have focused on examining the antioxidant properties of pirfenidone *in-vitro*. It has been reported that pirfenidone can reduce reactive oxygen species generation by activated neutrophils and macrophages and TGF-β-stimulated murine mesangial cells.[21,22] Pirfenidone also has been found to decrease the level of lipid peroxidation. Different studies have been done on the biochemical level to define the mechanism of the antioxidant activity of pirfenidone, and all have reached the same results. Two studies suggested that pirfenidone turns off the hydroxyl radical generated by Fenton chemistry (the reaction of hydrogen peroxide with ferrous iron). However, there is still controversial information regarding the ability of pirfenidone to complex iron and eventually stop the generation of hydroxyl radical instead of acting as a radical quencher.[23] Regarding the ability of pirfenidone to turn off superoxide anion, three reports claimed that this effect is either not related to pirfenidone, it is not a prominent effect.[23,24] The current study shows a significant degree of prevention using pirfenidone (300 mg/kg) as a gastro-protective medication due to the above-explained mechanism. Macroscopically, pirfenidone showed a normal-like stomach with a significant decrease of ulcer number and severity, compared with the diclofenac group and very similar to the results of the omeprazole group. Histopathologically, pirfenidone showed a very close to normal view, with some epithelial cells erosion, normal parietal cell distribution, normal gastric pit, and normal surface epithelium with a few detached superficial epithelial cells, compared to the diclofenac group. Due to the lack of any pre-clinical or clinical study using pirfenidone in GU, the above obtained results of pirfenidone use as a protective medication against GU came in agreement with, in which they found a similar results using pirfenidone in lung ischemia and due to the same mechanism.[25] The significant decrease in MPO and MDA activity in rats pre-treated with pirfenidone, on the other hand, omeprazole is an apparent indicator of anti-peroxidative and hence antioxidative potential. In comparison to the diclofenac group, pirfenidone is similar to omeprazole, and significantly reduced TNFα expression this may be due to anti-inflammatory action of pirfenidone *via* reducing pro- inflammatory cytokines. This is in agreement with (Bozkurt, I. *et al.,* 2022), this experimental study evaluated the effect of pirfenidone, for its anti-fibrotic, anti-inflammatory, and antioxidative properties.[26]

CONCLUSION

Diclofenac can be reduced or prevented by the pretreatment of pirfenidone. Pirfenidone showed similar results to the standard treatment (Omeprazole), the protective effect of pirfenidone was mainly through their anti-oxidant and anti-inflammatory activity by reducing oxidation markers like MPO and MDA, and also reducing inflammatory cytokines like TNF-alpha.

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