American Journal of Pharmacology and Toxicology 8 (3): 107-119, 2013
ISSN: 1557-4962

doi:10.3844/ajptsp.2013.107.119 Published Online 8 (3) 2013 (http://www.thescipub.com/ajpt.toc)

Sitagliptin Impairs Healing of Experimentally Induced Gastric Ulcers Via inhibition of iNOS and COX-2 Expression

[1,2]Amina Unis and [3]Eman Abdelzaher

[1]Department of Pharmacology, Faculty of Medicine, Tabuk University, Saudi Arabia
[2]Department of Pharmacology, Faculty of Medicine, Alexandria University, Egypt
[3]Department of Pathology, Faculty of Medicine, Alexandria University, Egypt

Received 2013-07-18, Revised 2013-08-17; Accepted 2013-08-22

ABSTRACT

Gastric ulcer healing is a complex process that is regulated by several promoting factors including COX-2 and iNOS. Diabetes mellitus is usually associated with delayed gastric ulcer healing. Hence, the current study was designed to investigate the effect of sitagliptin (dipeptidyl peptidase-4 inhibitor) on gastric ulcer healing and expression of iNOS and COX-2 in rat stomach.The study was conducted on 30 rats divided into three equal groups. Group 1 served as normal control group. Gastric ulcer was induced, by serosal application of acetic acid, in group 2 (ulcer model group) and group 3 (sitagliptin-treated group). Sitagliptin was administrated from day 3 to day 10 in group 3. All rats were sacrificed on day 10 and stomachs were removed for pathological examination and immunohistochemical assessment of COX-2 and iNOS expression. Pathological examination revealed that gastric ulcer healing was significantly impaired in the sitagliptin-treated group as evidenced by the significantly larger ulcerated area and ulcer base maturation impairment.COX-2 and iNOS expression as well as mean MVD were significantly diminished in the sitagliptin-treated group as compared to the ulcer model group. A significant positive correlation was found between COX-2 and iNOS implying their synergistic action. We conclude that sitagliptin significantly impairs gastric ulcer healing in rats possibly through inhibition of iNOS and COX-2 expression. Our results raise the question of whether sitagliptin is advisable in diabetic patients with pre-existing gastric ulcer. Our preliminary experimental findings need to be substantiated by future human studies.

Keywords: Sitagliptin, Gastric Ulcer Healing, Inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase-2 (COX-2), Microvessel Density (MVD)

1. INTRODUCTION

Gastric ulcer is considered one of the most prevalent gastrointestinal disorders. Its clinical outcome is determined by its liability to heal in order to prevent further damage to the gastric mucosa (Dharmani *et al*., 2003; Xie *et al*., 2013).

Gastric ulcer healing is a dynamic process encompassing epithelial regeneration, angiogenesis and maturation of the base (reduction of the ulcer base size) and is regulated by multiple factors (Tarnawski *et al*., 1995; Sato *et al*., 2013). COX-2 (cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) are among the most important healing-promoting factors for gastric ulcer (Shigeta *et al*., 1998; Tatemichi *et al*., 2003; Chatterjee *et al*., 2013). COX-2 induces the synthesis of Prostaglandins (PGs) that have stimulatory effects on ulcer healing (Takahashi *et al*., 1998a). iNOS-derived Nitric Oxide (NO) contributes to gastric ulcer healing through maintenance of an increased blood flow at the ulcer margin and stimulation of angiogenesis in the ulcer base as well as inhibition of inflammatory neutrophil accumulation via downregulation of surface expression of adhesion molecules (Salzman *et al*., 1998; Konturek *et al*., 1993). Recently, it was shown that the iNOS-based

Corresponding Author: Amina Unis, Department of Pharmacology, Faculty of Medicine, Alexandria University, Egypt

inflammatory pathway cross-link with the more well-known COX-2 pathway. This synergistic molecular interaction between the two inflammatory systems may cast more light on their healing promoting effects on gastric ulcer (Kim *et al*., 2005).

Diabetic patients are more vulnerable to develop gastric ulcers as diabetes leads to impairment of the antioxidant defense system of the gastric mucosa (Owu *et al*., 2012; Konturek *et al*., 2010). In addition, diabetic patients with gastric ulcers may suffer from reduced perception of the typical gastrointestinal symptoms due to diabetic neuropathy and they are at increased risk of bleeding (Boehme *et al*., 2007). Furthermore, diabetes may be associated with delayed healing of gastric ulcer due to significant decrease in the gastric microcirculation possibly resulting from reduction in mucosal prostaglandins (Brzozowska *et al*., 2004). Moreover, it was reported that hyperglycemia together with the increased production of proinflammatory cytokines result in sustained inflammatory reaction and thus may be responsible for the delay of healing at the ulcer area (Cosentino *et al*., 2003). Such previously stated reports necessitate studying the effect of antidiabetic drugs on gastric ulcer healing.

Dipeptidyl Peptidase-4(DPP-4) inhibitors are recently introduced drugs used for treatment of type 2 diabetes. Recent studies demonstrated that DPP-4 inhibitors or related compounds may possess marked inflammatory modifying effects through modulation of cytokine production (Alonso *et al*., 2012).

To the best of our knowledge, there have been no studies in the literature investigating the effect of DPP-4 inhibitors on gastric ulcer healing. Accordingly, the purpose of this research was to explore the effect of oral administration of sitagliptin (DPP-4 inhibitor) on the healing process of experimentally induced gastric ulcer in rats. In addition, the relation between sitagliptin and expression of healing-promoting factors (COX-2 and iNOS) was also investigated.

2. MATERIALS AND METHODS

2.1. Experimental Animals

All experiments were performed in accordance with national animal care guidelines and were preapproved by the Ethics Committee at Faculty of Medicine, Alexandria University.

The present study was conducted on 30 male Wistar albino rats weighing from 150 to 200 g. The rats were obtained from the Animal House at the Faculty of Medicine, Alexandria University. They were housed under optimal laboratory conditions (relative humidity 85±2%, temperature 22±1°C and 12 h light and 12 h dark cycle). All through the study, rats were fed on standard commercial pellet diet and had free access to drinking water.

2.2. Animal Grouping

Rats were divided into 3 groups of 10 rats each:

Group 1: (normal control group) in which rats had free access to drinking water without any additive.

Group 2: (gastric ulcer model) in which gastric ulcer was induced in rats and they had free access to drinking water without any additive.

Group 3: (sitagliptin-treated group) in which rats received sitagliptin added to the drinking water ,at a dose of 30 mg/kg orally every day, beginning on day 3 and continuing for 7 days following gastric ulcer induction. The dose of 30 mg/kg/d is considerably higher than the human dose because sitagliptin has a half-life of two hours in rats (Beconi *et al*., 2007) versus 13 h in humans (Dhillon, 2010). This short half-life necessitated continuous administration through drinking water instead of the once-a-day dosing used in humans (Chen *et al*., 2011). The Institutional Animal Care and Use Committee (IACUC) protocol of Boston University-USA for adding a novel compound to the drinking water was followed in order to ensure that each rat received the exact dose in the drinking water (IACUC, 2013).

2.3. Induction of Gastric Ulcer

After fasting for 18 h, rats were anesthetized, using halothane and gastric ulcers were induced by application of 0.2 mL of acetic acid (100%) to the serosal surface for 60 sec as described by Okabe and Amagase (2005). This model of gastric ulcer was chosen as it highly resembles human ulcers in terms of both pathological features and healing process.

Ten days following gastric ulcer induction, rats were sacrificed by an overdose of intraperitoneally injected sodium pentobarbital. The stomachs were removed, opened along the greater curvature and rinsed with saline then they were fixed in 10% buffered formalin.

2.4. Pathological Assessment of Ulcer Healing

The stomachs were grossly examined for pathological changes. The ulcerated area (mm^2) was quantified using the following equation:

$$S = \pi (d1/2)X(d2/2)$$

where, S represented the ulcerated area (mm^2), d1 and d2 the longest longitudinal and transverse diameters of the ulcer (Kang *et al*., 2010).

Representative sections were routinely processed. 5 µm-thick sections were cut and stained with the conventional Haematoxylin and Eosin (H&E) stain and examined by the light microscope for histopathological assessment. Masson trichrome stain was used to highlight fibrosis. The degree of inflammation, degeneration and thickness (maturation) of ulcer base were semi-quantitatively assessed at the ulcer bed. Length of regenerated mucosa (mm) was also measured.

2.5. Immunohistochemistry for iNOS and COX-2

The deparaffinized tissue sections were rehydrated in graded alcohols. Immunohistochemical staining was performed using an avidin-biotinylated immunoperoxidase methodology. The endogenous peroxidase activity was quenched by using hydrogen peroxide 3% for 10 min. For antigen retrieval, sections were microwaved in 10mM citrate buffer (pH 6.0). Prediluted primary antibodies, COX-2 (clone SP21, rabbit monoclonal antibody) and iNOS (rabbit polyclonal antibody) were used. The bound antibodies were detected by the UltraVision Detection System Anti-Polyvalent, HRP/DAB (Ready-To-Use). Positive and negative controls were included in all runs.

Primary antibodies and detection system were purchased from Lab Vision Corporation, Thermo Fisher Scientific Inc., USA.

2.6. Computerized Image Analysis (CIA)

Quantitative estimation of the total area of positive reaction was done on histological sections immunostained for iNOS and COX-2 using image analyzer software (Digimizer ® Version 4.1, MedCalc Software, Belgium).

Binary images for measurement were generated and the mean total area of positive reaction was calculated.

2.7. Assessment of Microvessel Density (MVD)

Sections were immunostained by the vascular marker, CD31 (rabbit polyclonal antibody) as described above. (**Fig. 1d**) MVD was then calculated as previously described (Dai *et al*., 2005).

2.8. Statistical Analysis

Data were analyzed using Statistical Package for Social Science (SPSS® Statistics 20). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test. Quantitative normally distributed variables were described using mean and standard deviation. Independent t-test was used to compare their means. Both quantitative abnormally distributed and Qualitative ordinal variables were described using median, minimum and maximum. Correlations were tested using Spearman's correlation coefficient. Mann-Whitney (U) test was used to compare their distributions. Statistical Significance was judged at the 5% level ($p \leq 0.05$).

3. RESULTS

3.1. Gastric Ulcer Model Showed Significant Pathological Changes and Increased MVD

Gastric serosal application of acetic acid in rats resulted in statistically significant pathological changes in ulcer model group (**Fig. 1b**) compared to normal control group (**Fig. 1a**): mean ulcerated area (mm^2) ($p<0.001$), degenerated mucosa ($p<0.001$), inflammatory exudates ($p<0.001$), thickness of ulcer base ($p<0.001$) and length of regenerated mucosa (mm) ($p<0.001$).

MVD was significantly higher in the ulcer model group compared to the normal control group ($p<0.001$). In addition, the length of regenerated mucosa (mm) was significantly positively correlated with MVD ($\rho = 0.532$, $p = 0.003$).

3.2. Induction of Gastric Ulcer Significantly Induced COX-2 and iNOS Expression

COX-2 and iNOS expression were induced in the stomachs of ulcer model group (**Fig. 2a and b**) with a statistically significant higher expression compared to the normal control group that lacked their expression ($t = 6.90$, $p<0.001$ and $t = 5.79$, $p<0.001$ respectively). COX-2 and iNOS were most intensely expressed in inflammatory cells at the ulcer base (**Fig. 2a and b**).

3.3. Sitagliptin Impaired Gastric Ulcer Healing and Significantly Inhibited COX-2 and iNOS Expression and Diminished MVD

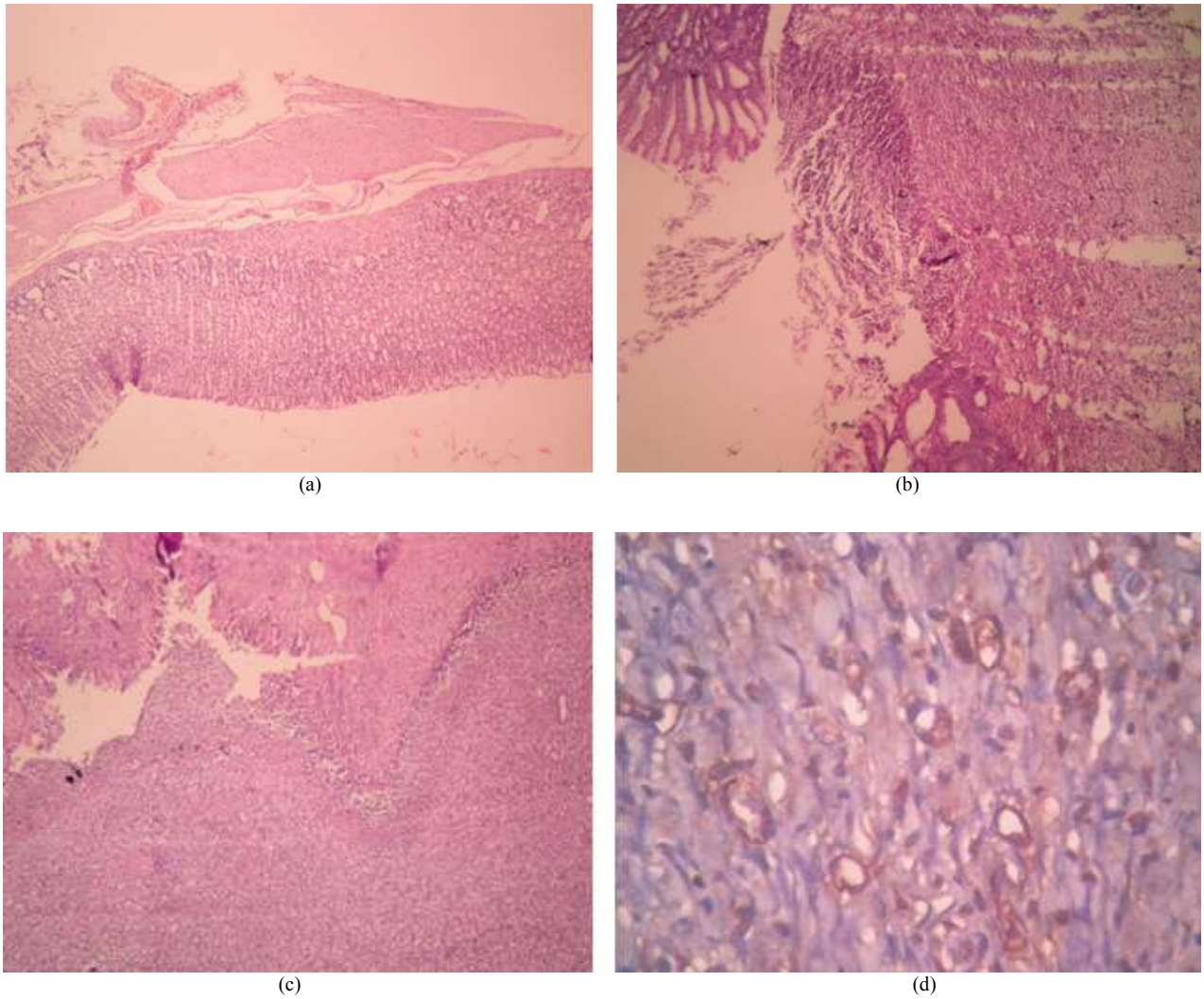
Seven days treatment with sitagliptin in group 3 resulted in pathologically proven significant impairment of gastric ulcer healing (**Fig. 1c**) as compared to the ulcer model group (**Fig. 1b**). The ulcerated area in the sitagliptin-treated group was significantly larger (nearly 9 times wider) than the model group ($U = 2.50$, $p = 0.001$) (**Table 1 and Fig. 3a**).

COX-2 and iNOS expression as well as MVD were significantly diminished in the sitagliptin-treated group compared to the model group ($t = 3.84$, $p = 0.001$); ($U = 8.00$, $p = 0.001$); and ($t = 5.55$, $p<0.001$) respectively. (**Fig. 3b,c,d and Table 1**).

The expression of COX-2 and iNOS in the sitagliptin-treated group (**Fig. 2c and d**) was more pronounced at the ulcer margins with less intense expression in inflammatory cells at the ulcer base.

The mean ulcerated area (mm^2) was significantly negatively correlated with COX-2 expression ($\rho = -0.652$, $p = 0.002$); iNOS expression ($\rho = -0.702$, $p = 0.001$); and MVD ($\rho = -0.635$, $p = 0.004$) (**Fig 4a, b, c**).

Maturation of ulcer base was significantly impaired ($U = 20$, $p = 0.023$) in the sitagliptin-treated group compared to the model group. **Table 1** in addition, it was significantly negatively correlated with COX-2 expression ($\rho = -0.508$, $p = 0.026$); and iNOS expression ($\rho = -0.548$, $p = 0.015$).



(a) (b) (c) (d)

Fig. 1. Histopathological changes in the studied groups: (a): Normal control group showing intact mucosal surface with absent inflammation and fibrosis (H&E, 40x). (b): Gastric ulcer model; showing ulcerated area of moderate size and the ulcer base is covered by necroinflammatory debris (H&E, 40x). Sitagliptin-treated group showing (c) large-sized ulcer with thickened ulcer base and intense inflammation (H&E, 40x); (d) (d) Microvessels highlighted by CD31 immunostain. (200x)

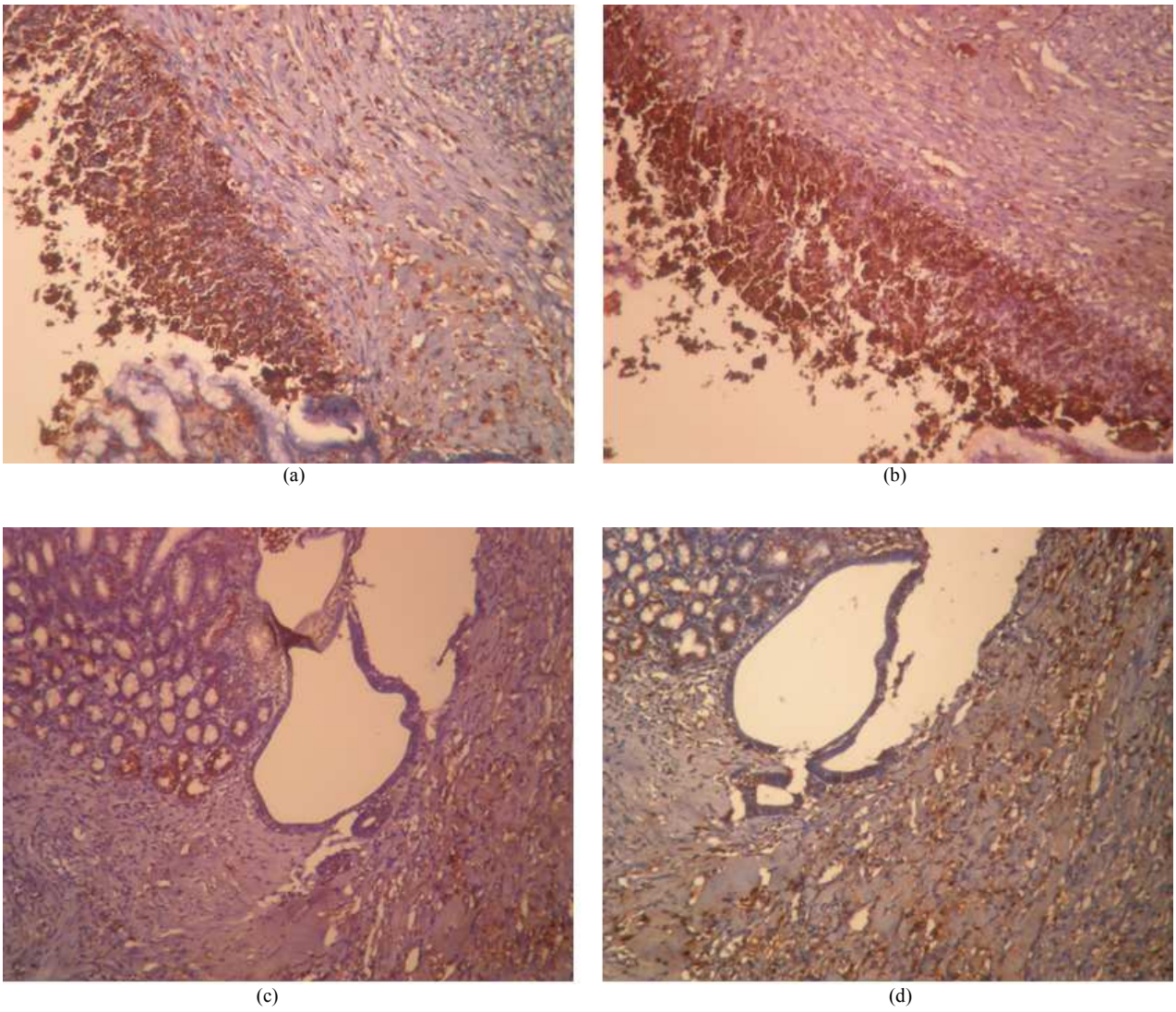
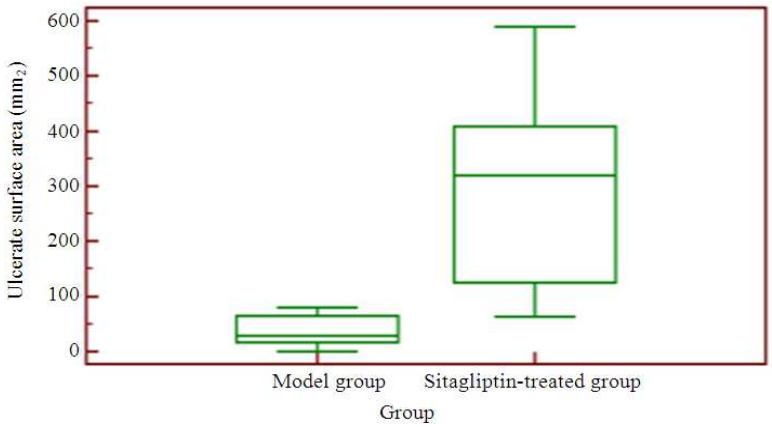


Fig. 2. Immunohistochemical expression of iNOS and COX-2 in the studied groups under 100x original magnification: Upper panel: Acid-induced gastric ulcer model showing intense iNOS (a) and COX-2 (b) expression in the ulcer base. Lower panel: Sitagliptin-treated group showing diminished iNOS (c) and COX-2 (d) expression in ulcer base with moderate expression at the ulcer margins

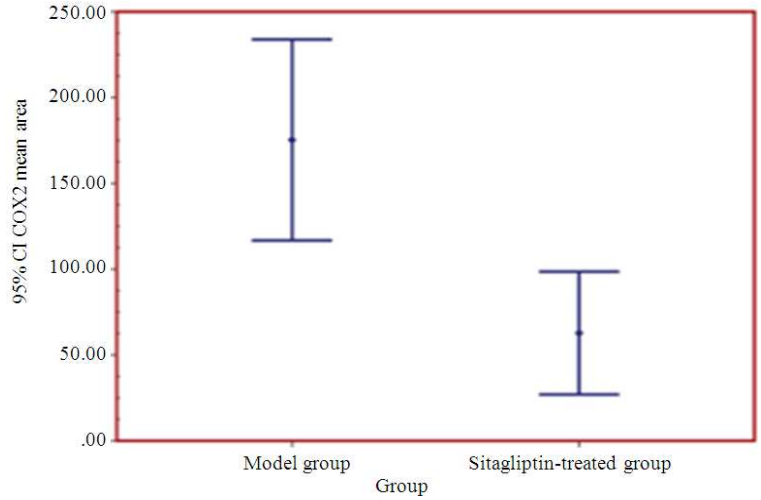
Although inflammatory changes (intensity of inflammatory exudate and mucosal degeneration) were severer and mucosal regeneration was less pronounced in the sitagliptin-treated group compared to the ulcer model group, the results did not reach statistical significance (U = 30.5, p = 0.143); (U = 33.00, p = 0.218) and (U = 23.00, p = 0.079) respectively. However, the intensity of inflammatory exudate was significantly negatively correlated with COX-2 expression (ρ = -0.477, p = 0.039); and iNOS expression (ρ = -0.507, p = 0.027).

3.4. Positive Correlation Between iNOS, COX-2 and MVD

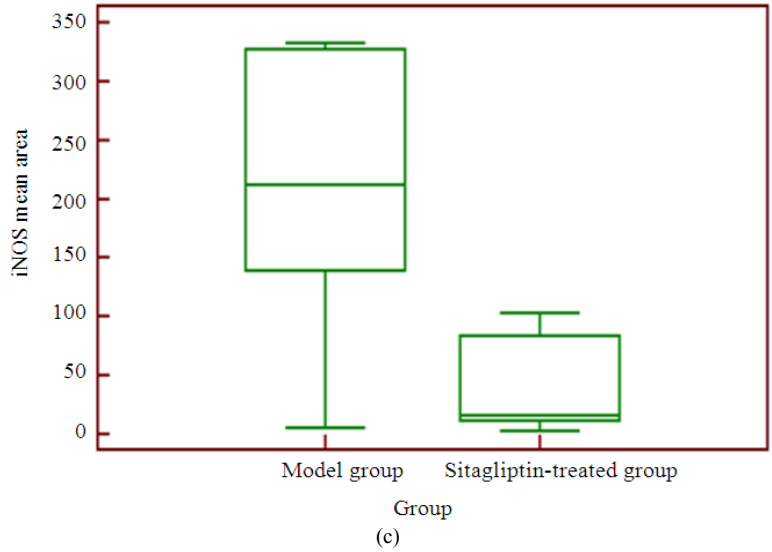
Our study showed a statistically significant positive correlation between COX-2 and iNOS expression (ρ = 0.989, p = <0.001) (**Fig. 4d**).


Ulcerate surface area (mm2)
600
500
400
300
200
100
0
Model group
Sitagliptin-treated group
Group

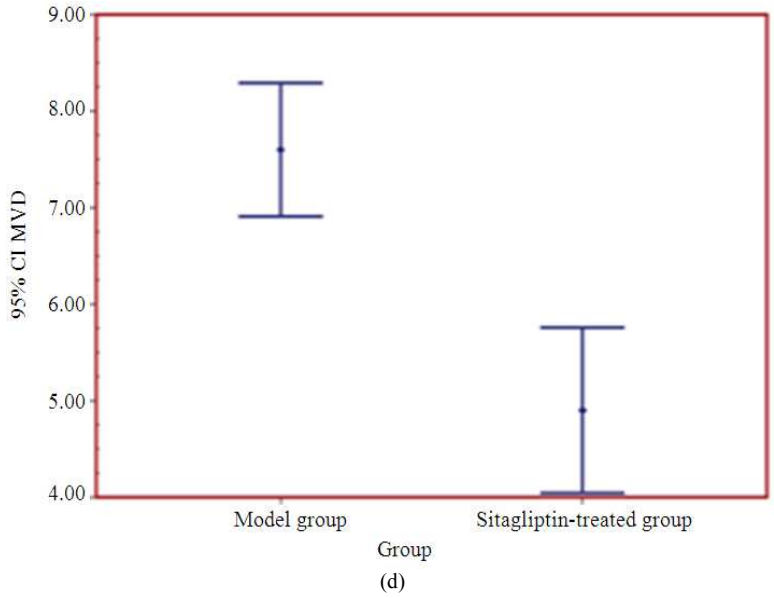
(a)


95% CI COX2 mean area
250.00
200.00
150.00
100.00
50.00
.00
Model group
Sitagliptin-treated group
Group

(b)


iNOS mean area
350
300
250
200
150
100
50
0
Model group
Sitagliptin-treated group
Group

(c)



(d)

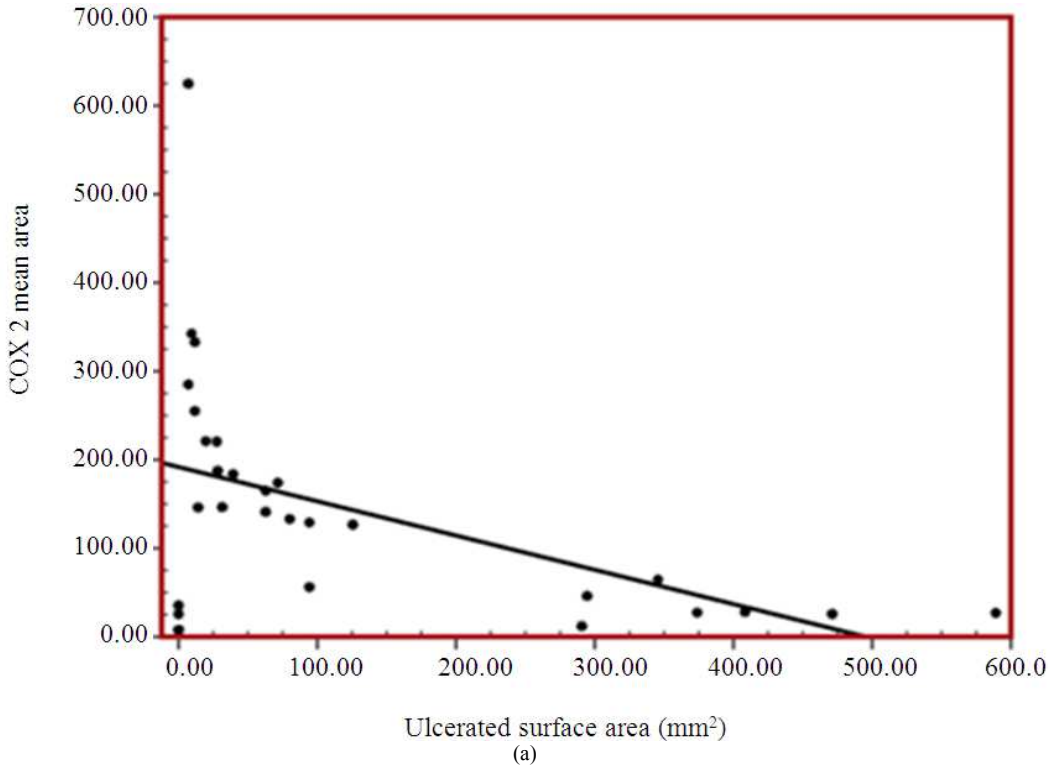
Fig. 3. Comparisons between the model group and the sitagliptin-treated group regarding the mean ulcerated area (a), COX-2 expression (b), iNOS expression (c) and MVD (d). The ulcerated area in the sitagliptin-treated group was significantly larger than the model group (U = 2.50, p = 0.001). COX-2 and iNOS expression as well as MVD were significantly diminished in the sitagliptin-treated group compared to the model group (t = 3.84, p = 0.001); (U = 8.00, p = 0.001) and (t = 5.55, p<0.001) respectively

Table 1. Comparison between the ulcer model group and sitagliptin-treated group regarding all the studied variables

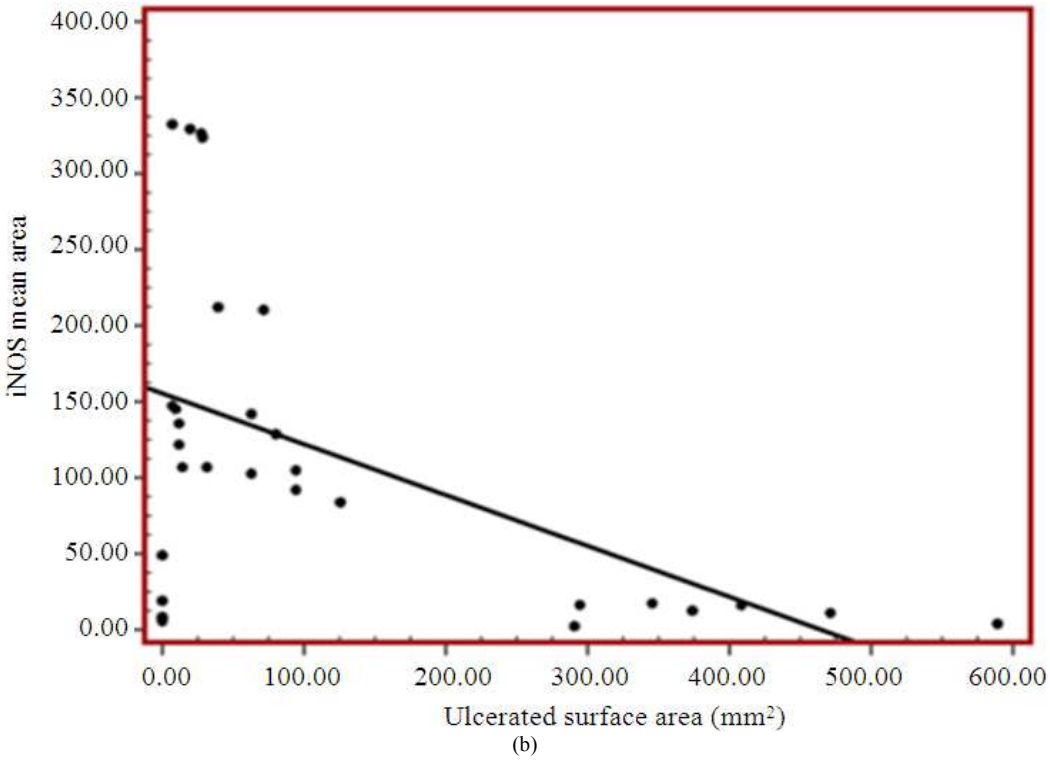
Group variable	Gastric ulcer model group	Sitagliptin-treated group	Test statistic (p-value)
Ulcerated area (mm^2)			U = 2.50
Mdn (Min-Max)	33.77(0.00-435.90)	320.05(62.83-589.05)	(0.001)*
Degenerated mucosa			U = 33.00
Mdn (Min-Max)	1(0-3)	2(1-3)	(.218)
Inflammatory exudate			U = 30.5
Mdn (Min-Max)	2(1-3)	2(1-3)	(.143)
Thickness of ulcer base			U = 20
Mdn (Min-Max)	2(1-3)	3(1-3)	(0.023) *
Length of regenerated mucosa (mm)			U = 23.00
Mdn (Min-Max)	4.00(0.00-20.00)	0(0.00-5.00)	(.079)
iNOS mean area			U = 8.00
Mdn (Min-Max)	211.21(5.70-332.49)	16.22(2.30-102.61)	(0.001) *
COX2 mean area			t = 3.84
M±SD	175.29±76.15	62.79±50.08	(0.001) *
MVD			t = 5.55
M±SD	7.60±0.97	4.90±1.20	(<0.001) *

Mdn: Median, M: mean, SD: standard deviation, U: Mann-Whitney, *: significant

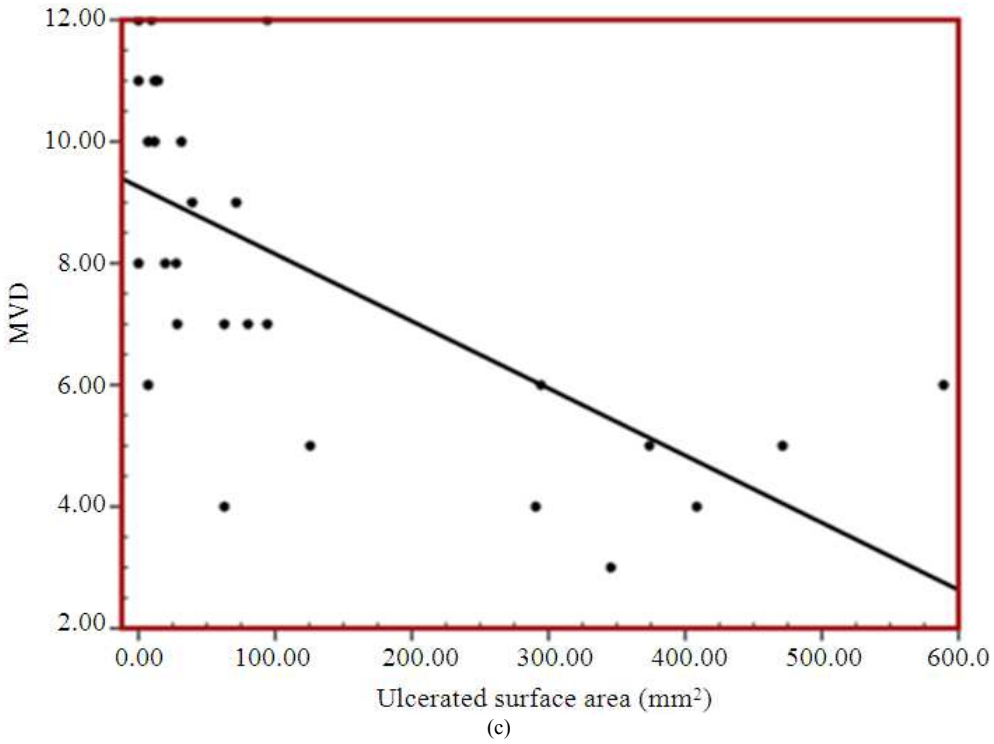
A statistically significant positive correlation was also found between COX-2 expression and MVD (ρ = 0.510, p = 0.026) on one hand and iNOS expression and MVD (ρ = 0.540, p = 0.017) on the other hand.


700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
COX 2 mean area
0.00
100.00
200.00
300.00
400.00
500.00
600.0
Ulcerated surface area (mm²)

(a)


400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
iNOS mean area
0.00
100.00
200.00
300.00
400.00
500.00
600.00
Ulcerated surface area (mm²)

(b)



(c)

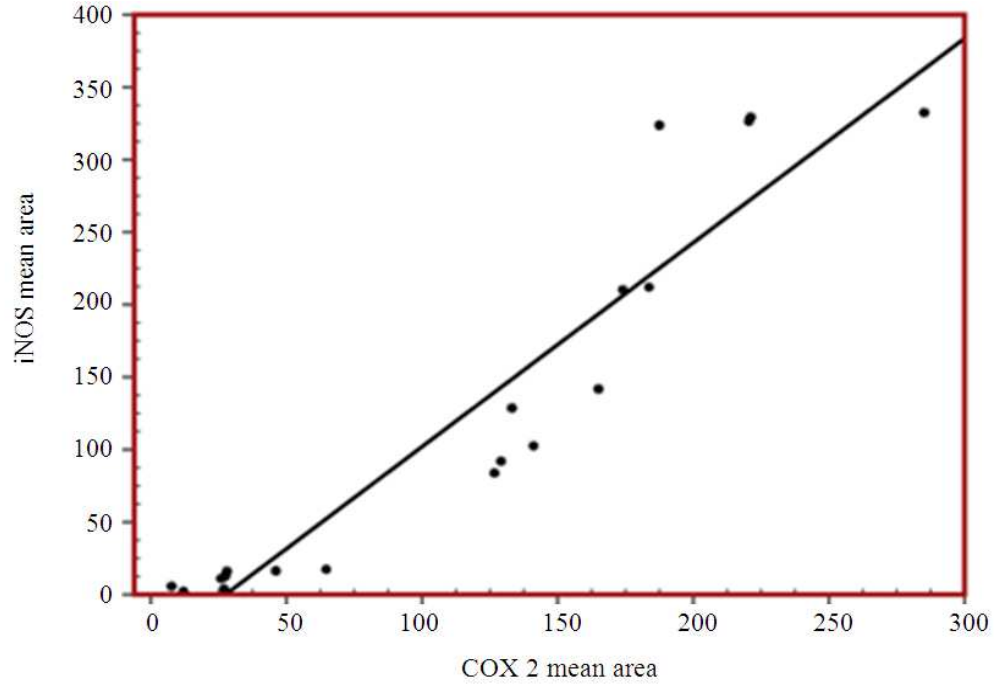


Fig. 4. The mean ulcerated area (mm^2) was significantly negatively correlated with (a): COX-2 expression (ρ = -0.652, p = 0.002); (b): iNOS expression (ρ = -0.702, p = 0.001); and (c): MVD (ρ = -0.635, p = 9.004). (d): A statistically significant positive correlation between COX-2 and iNOS expression (ρ = 0.989, p=<0.001)

4. DISCUSSION

Gastric ulcer refers to a disruption of the mucosal integrity of the stomach with local excavation due to active inflammation (Valle, 2002). iNOS and COX-2 represent important lines of defense necessary for maintenance of mucosal integrity and are important factors in ulcer healing processes especially angiogenesis, base maturation and modulation of inflammatory reactions (Takahashi *et al*., 2001; Stachura *et al*., 1995; Akiba *et al*., 1998; Dharmani *et al*., 2003).

In addition, iNOS-derived NO and COX-2 derived PGs have been reported to have an impact on a wide variety of cell types and processes that may be active during inflammatory responses, including leucocyte adhesion and microvascular responsiveness hence they play important roles in gastric ulcer healing (Mizuno *et al*., 1997; Hickey, 2001).

Moreover Allen *et al*. (1988) proposed that NO plays an important role in ulcer healing by forming a gelatinous coat covering the ulcer bed, consisting of a fibrin-based gel with mucus and necrotic cells, which acts as a protective barrier preventing direct contact with the gastric luminal contents. Furthermore, Wallace (2008) highlighted the fact that the protective functions of PGs in the stomach can be carried out by other mediators, in particular NO.

COX-2 and iNOS are normally undetectable in most normal tissues, their expression being induced only at inflammatory sites (Mitchell *et al*., 1995; Okazaki *et al*., 2007). The results of the present study are in accordance with that finding, as normal control stomach tissues lacked expression of both markers.

On the other hand, significant expression of COX-2 and iNOS was detected in ulcer bed in the model group when rats were sacrificed 10 days after gastric ulcer induction. In agreement with our study, Tatemichi *et al*. (2003) and Shigeta *et al*. (1998) stated that iNOS and COX-2 expression peaked during the rapid healing phase and were limited to ulcer bed. According to Halter *et al*. (1995) four healing phases are recognized in experimental models of gastric ulcer: an early lag phase (days 1-3), a rapid healing phase (days 3-14), a late lag phase (days 14-18) and a remodeling phase (day 18 and onward). In the ulcer model group in our study, COX-2 and iNOS expression was mainly encountered in inflammatory cells at ulcer bed. Similarly, Shigeta *et al*. (1998) reported that strong COX-2 immunoreactivity was found in macrophages/monocytes, granulocytes and fibroblasts at ulcer bed. Also, Tatemichi *et al*. (2003) demonstrated that iNOS-positive cells were localized only among the inflammatory cells and fibroblasts at ulcer bed.

Angiogenesis is an another important factor that play a pivotal role in gastric ulcer healing since the neovasculature promotes nutrient supply to the healing tissue (Takahashi *et al*., 1998b). In the present study, MVD (one of most commonly used techniques to quantify angiogenesis according to (Kang *et al*., 2010) was significantly increased in the model group and was significantly positively correlated with the length of regenerated mucosa. In addition, a positive correlation was detected between iNOS and COX-2 expression on one hand and MVD on the other hand. Such findings suggest that iNOS and COX-2 may contribute to ulcer healing process through regulation of angiogenesis. This was further supported by Konturek *et al*. (1993) who reported that NO stimulates angiogenesis in the ulcer base, contributing to gastric ulcer healing. Also, Leahy *et al*. (2000) stated that COX-2-derived PGs have similar angiogenic stimulating effects.

In the current study, a statistically significant positive correlation between COX-2 and iNOS expression was detected. Such finding further supports the recent identification of a synergistic molecular interaction between COX-2 and iNOS pathways proving that these two systems are related and may represent a major mechanism in inflammatory responses (Kim *et al*., 2005; Fang *et al*., 2000; Kornau *et al*., 1995).

As diabetes is associated with delayed ulcer healing the present study examined the effect of one of the recently introduced oral antidiabetic drugs sitaglitpin (DPP-4 inhibitor) on healing process of gastric ulcer. DPP-4 is a serine protease that is widely distributed throughout the body, expressed as an ectoenzyme on endothelial cells, on the surface of T-lymphocytes and in a circulating form. Although there are many potential substrates for this enzyme, it seems to be especially critical for the inactivation of incretin hormones: GLP-1 (glucagon like peptide -1) and Gastric Inhibitory Peptide (GIP) (Baggio and Drucker, 2007).

In our study, ulcer healing was significantly impaired in the sitagliptin-treated group. Compared to the ulcer model group, the ulcerated area in the sitagliptin-treated group was significantly larger and maturation of ulcer base was significantly impaired. In addition, inflammatory changes were severer and mucosal regeneration was less pronounced in the sitagliptin-treated group compared to the ulcer model group, however, these results did not reach statistical significance.

Expression of COX-2, iNOS and MVD in our study were significantly diminished in the sitagliptin-treated group compared to the ulcer model group. This was further substantiated by our finding of a significant negative correlation between the mean ulcerated area on one hand and COX-2 expression, iNOS expression and MVD on the other hand. In addition, the intensity of inflammatory changes and thickness (maturation) of ulcer base in our study were significantly negatively correlated with COX-2 and iNOS expression.

Such results suggest that sitagliptin acts as inhibitor of both COX-2 and iNOS leading to impairment of ulcer healing processes specially angiogenesis. This is in accordance with (Tatemichi *et al*., 2003; Shigeta *et al*., 1998) who reported that administration of COX-2 and iNOS inhibitors resulted in significant prevention of mucosal regeneration and maturation of the ulcer base as well as regression of angiogenesis in the examined rat stomachs.

In the sitagliptin-treated group in our study, COX-2 and iNOS were mostly expressed at the ulcer margins with less intense expression at the ulcer base which probably has a deleterious effect on ulcer healing. This is in accordance with Tarnawski *et al*. (1995) who reported that iNOS were to act detrimentally on ulcer healing if it is expressed at the ulcer margin which is an important area for ulcer healing, supplying new epithelial cells (regenerating zone).

Few reports have investigated the effect of sitagliptin administration on iNOS expressions in various tissues. Nader *et al*. (2012) have shown that NO content as well as the mRNA expression of iNOS was remarkably decreased by sitagliptin treatment in murine model of allergic airway disease.

Other studies investigated the role of incretins and incretin mimetics on iNOS expression. Salehi *et al*. (2008) reported that GLP-1 suppressed excessive NO generation and iNOS activity in diabetic rat islets via the activation of cAMP/PKA system. Also, (Belin *et al*., 1999; Jimenez-Feltstrom *et al*., 2005) demonstrated that GLP-1 reduced NO production through increasing the level of cAMP in high glucose- and IL-1β-stimulated islets respectively. In addition, (Li *et al*., 2005; Kang *et al*., 2009) showed that exenatide (GLP-1 agonist) decreased cytokine-induced iNOS protein expression.

On the other hand, Ye *et al*. (2010) have shown that sitagliptin had no effect on COX-2 activity in experimentally induced myocardial infarction in rats.

5. CONCLUSION

The findings of our study together with previous reports show that the acetic acid-induced gastric ulcer model serves as an excellent model for the study of gastric ulcer development and healing. In addition, it provides further evidence on the synergistic actions of COX-2 and iNOS and the fact that they contribute to gastric ulcer healing possibly through stimulation of angiogenesis and modulation of inflammatory responses.

Sitagliptin was found to significantly impair gastric ulcer healing in rats, possibly through inhibition of iNOS and COX-2 expression. Thus further studies are needed to justify its prescription to diabetic patients with preexisting gastric ulcer.

The cellular mechanism by which sitagliptin inhibits iNOS and COX-2 expression in the ulcerated gastric mucosa remains to be elucidated.

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