Possible Protective Effect of Each of Omega-3 PUFA and Leptin on Indomethacin-Induced Gastric Ulcer in Rats with type II DM

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Abstract

Background: Patients with diabetes mellitus (DM) are at significant risk for variable complications particularly, gastrointestinal tract disorders such as peptic ulcers. Omega-3 polyunsaturated fatty acids (PUFAs) and leptin can help tissue healing through their anti-inflammatory, antidiabetic, and antioxidant effects. This work compares ω-3 PUFAs and leptin impact on gastric ulcers in diabetics. **Methods:** 36 rats were divided equally into negative control (n = 9) and Streptozotocin-induced type-II DM (n = 27) groups. The latter group was subdivided into three equal subgroups (n=9): diabetic control, diabetic with leptin, and diabetic with ω-3 PUFAs. A single oral indomethacin dose induced the ulcer by the end of the experiment. We assessed gastric mucosa gross appearance, histopathological changes and biochemical parameters. **Results:** Chronic administration of leptin and ω-3 PUFAs remarkably ameliorated gastric ulcer index, ulcer protection percentage, the gastric expression of reduced glutathione, cholecystokinin and endothelial nitric-oxide synthase genes, and significantly decreased the expression of proton pump gene and cyclo-oxygenase-2 enzyme activity. **Conclusion:** This work found that leptin and ω-3 have antacid, anti-inflammatory, and antioxidant effects against indomethacin-induced gastric ulcers in diabetic rats, with a more potent effect of ω-3. We hypothesize that concomitent use of both would have an augmented anti-ulcer results.

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INTRODUCTION

Diabetes mellitus (DM) is a widely-spread metabolic disorder characterized by increased production of reactive oxygen species (ROS) with a concomitant decreased antioxidant capacity [1]. ROS in low to moderate levels is physiologically beneficial to various healing and repair processes. Disproportionate ROS production alters bodily homeostatic mechanisms with oxidative tissue damage [2]. Diabetics are at increased hazard for developing numerous vascular dysfunctional complications affecting the nervous system, the heart, the blood vessels and the gastrointestinal tract (GIT) [3]. Nevertheless, people with diabetes are more vulnerable to developing acute gastritis with or without peptic ulcers as a result of increased mucosal vulnerability to various ulcerogenics [4]. Moreover, DM increases the susceptibility to gastroparesis and gastric ulcers risk due to long-standing exposure to gastric acid by the poorly protected gastric mucosa [5].

Gastric ulcer affects about 10% of the population worldwide [6]. Multiple endogenous and exogenous factors include acid, pepsin, stress, and harmful agents such as alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), *Helicobacter pylori* bacteria, and smoking are known to induce or aggravate already existing gastric ulcers [7]. Prolonged NSAID use inhibits cyclooxygenase (COX) enzymes which reduces prostaglandin (PG) synthesis which damages gastric mucosa and postpones ulcer healing. Another COX-independent mechanism also exists [8,9]. NSAIDs are extensively used to reduce pain and inflammation [10]. About 20%–30% of gastric ulcer patients take NSAIDs [11].

Ω-3 polyunsaturated fatty acids (PUFAs), also known as long-chain PUFAs, is found in various foods, including green-leafy plants, seafood, fish, and nuts [12]. PUFAs, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have numerous health benefits, and that is why they help treat multiple pathological conditions. EPA is converted into potent anti-inflammatory cytokines such as PGs, thromboxanes, and leukotrienes [13]. In addition, by lowering blood glucose levels and inflammation and improving insulin sensitivity, PUFAs are considered antidiabetic agents [14,15]. Ω-3 may also benefit in protecting gastric mucosa from injury and subsequent ulceration [16].

Leptin, the "satiety hormone", is a hunger inhibitory hormone that helps regulate energy balance. It is secreted by adipose cells. Ghrelin, which is sometimes refered to as "hunger hormone," has an opposite action of leptin. Both hormones act on hypothalamic arcuate nucleus receptors to regulate appetite and achieve energy homeostasis [17]. Decreased leptin sensitivity is usually an adverse finding in obesity with lost ability to detect satiety in spite of high energy stores [18].

Based on our information, a few pieces of literature discussed the beneficial role of leptin or ω-3 in preventing gastric ulcers. That is why our study aimed to investigate their potential protective effects in preventing indomethacin-induced gastric ulcers in streptozotocin-induced diabetic rats.

2. Methods

2.1. Experimental Animals:

Thirty-six male albino rats weighing 160 ±15 g were enrolled in this study obtained from Kasr Al-Aini Faculty of Medicine Animal House, Cairo University, Egypt. Before being enrolled in the experiment, animals we kept under observation for ten days for adaptation and any diseased or infected rats were excluded. The rats were placed in wire-mesh cages (50 x 20 x 20 cm, three/cage) at room temperature (25 ±5 °C), with well-ventilated covers and 12 hr alternating light/dark cycle. Animals had free access to ad-lib food and water. The Institutional Animal Care and Use Committee of Cairo University (CU - IACUC) approved the experimental study protocols, with approval number "CU/III/F/64/17". The experimental animals were then divided into two groups:

- **Group C** (negative control, n = 9): offered standard rodent food ad-lib for six weeks.

- **Group D** (diabetic control, n = 27): offered a short-term high-fat diet (HFD), (caloric content: fat 58.8%, carbohydrate 26.0%, protein 15.2%) for 14 days. This was followed by two subsequent intraperitoneal injections of low doses Streptozotocin (STZ) of 30 mg/kg in 0.01 mol/l citrate buffer (STZ; Sigma, St. Louis, USA) 24 hours apart. Rats included in the study as diabetic animals are those with FBG value ≥200 mg/dl five days after the second STZ injection, as measured by One Touch Ultra glucose meter, Life Scan, Milpitas, CA USA,

were [**19**]. Diabetic rats were further divided into three equal subgroups (n = 9 each):

- Subgroup Dc (diabetic control): offered a standard rodent diet for the study period.

- Subgroup Dl (leptin): offered a standard rodent diet enriched with leptin (10 µg/kg, SC) administered twice a day for the study period [**20**].

- Subgroup Df (PUFA): offered a standard rodent diet fortified with 100 g/kg ω-3 PUFA for the study period [**21**].

2.2. Design of indomethacin-induced gastric ulcer:

indomethacin (18 mg/kg, orally, single dose), dissolved in distilled water and suspended in 2% gum acacia as the vehicle was used to induce ulceration [21]. Two days after indomethacin administration, rats were sacrificed by the CO_2 euthanasia method [23]. Rats' stomachs were carefully removed and cut open along the greater curvatures.

2.3. Ulcer score:

by counting the ulcers using a magnifying hand lens (10X). Each lesion severity was scored in the following manner:

'1' → ulcers less than 1 mm (pinpoint)

'2' → ulcers 1-2 mm.

'3' → ulcers larger than 2 mm [**24**].

2.4. Ulcer protection percentage [25]:

was calculated from the formula = $(U_c-U_t/U_c) \times 100$

Where: U_c = control group ulcer index.

U_t = test group ulcer index.

Ulcer index could be obtained by dividing the ulcer score over 10.

2.5. Biochemical assays:

2.5.1. COX-2 activity: was measured colourimetrically as described previously [**26**].

2.5.2. Real-time PCR genes expression of eNOS, CCK, GSH and H⁺/K⁺-ATPase by: The gastric tissue homogenate was used to extract total RNA using Isolation Kit (Invitrogen, CA). By using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA), a single-strand cDNA was synthesized. Quantitative RT-PCR was performed using TaqMan Fluorescein one-step method with specific primers (**table 1**), according to **Kuang et al**. [**27**].

Table (1): The used oligonucleotide primers for PCR analysis

Genes	The used primer	References
eNOS	F* 5′-TCCGGAAGGCGTTTGATC-3′ R* 5′-GCCAAATGTGCTGGTCACC-3′	Won et al. 2007
CCK	F* 5′ GCGATTTGCAAACCCTTACAG-3′ R* 5′ CACCTTCAAAGCATGGGATTTT-3`	Kazmi et al. 2014
GSH	F* 5` GGAACGACAACCAGGGACTA 3` R* 5` TCCCTGGACGGACATACTTC  3`	El-Barbary 2015
H⁺/K⁺-ATPase	F* 5` CTTTGCCATCCAGGCTAGTGA 3` R* 5` CTTTGCCATCCAGGCTAGTGA 3`	Bao et al. 2020

2.6. Histopathological examination:
After quantifying ulcer score, stomachs were fixed in 10% neutral buffered formalin, processed and embedded in paraffin. **Hematoxylin and eosin** stain (H&E, Scytek Laboratories SDP Hematoxylin-Eosin Stain Kit, catalogue no. NC0510871) and **Masson's Trichrome** stain (Poly Scientific, Bay Shore, NY) were used to stain 4 µm thick sections in agreement with the manufacturer's protocols.

2.7. Immunohistochemical staining:
4 µm formalin-fixed, paraffin-embedded sections were deparaffinized in xylene, rehydrated, and stained with the anti-COX2 antibody [EP8588]-(ab169782)- Abcam according to the manufacturer's protocol. The appearance of brown staining denotes positive immunoreactions. The negative immunoreactivity of negative controls was performed by omitting the primary antibody. Reproducibility was ensured through testing sections from five different animals from each group and processing them simultaneously. The sections were examined and then photographed. A scale of 0 to 3 was used to calculate immunohistochemical results semi-quantitatively as follows [**28**].

0 🞂 absent

1 🞂 mild [10% of positive cells]

2 🞂 moderate [10 to 25%]

3 🞂 severe [25%]

2.8. Statistical Analysis: Windows ® statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL, USA) was used in the process of coding and entering data, which were summarized using mean and standard deviation (SD) for quantitative variables. The statistical differences between the data mean from different groups were analyzed using ANOVA with a post hoc test. P values were considered statistically significant if less than 0.05 and **highly** statistically significant if less than 0.01) [**29**].

3. **Results**

3.1. **The score of gastric ulcers and ulcer protection percentage:** As shown in figure (1-a and -b), the ulcer scores were highly statistically significantly ($p < 0.01$) greater in Dc (11.87 ± 0.69), statistically insignificantly ($p > 0.05$) greater in Dl (9.76 ± 0.69) and highly statistically significantly ($p < 0.01$) lower in Df (6.92 ± 0.95) subgroups compared to the C (8.96 ± 0.53) group, with -32.48%, -8.93 and +22.75% change in percentage ulcer protection, respectively. The figure also shows a highly statistically significantly ($p < 0.01$) reduction in the ulcer score of both Dl and Df subgroups compared to the Dc subgroup. Notably, ω-3 PUFA-fed animals had significantly more protection than leptin-fed animals ($p < 0.01$).
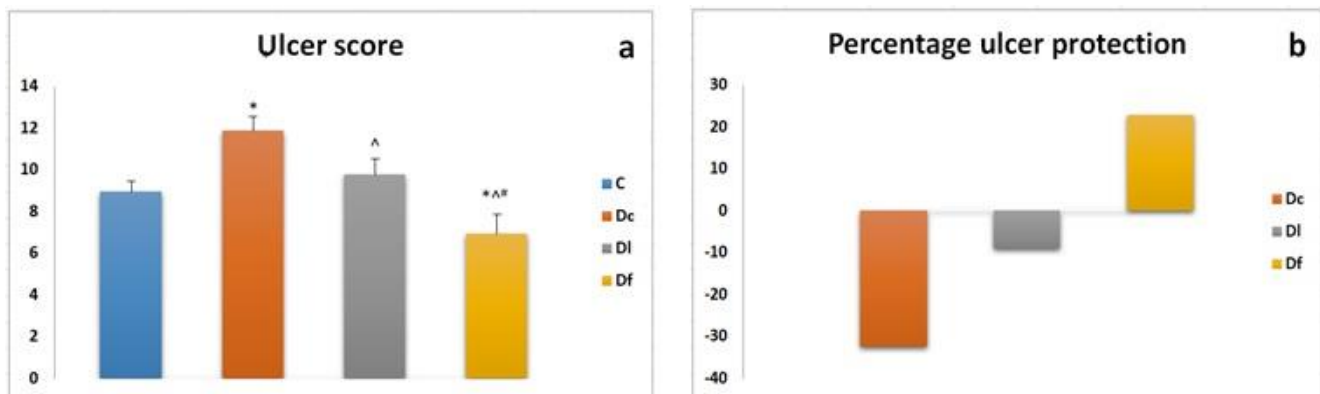

Figure (1): Ulcer scores (**a**) and percentage ulcer protection (**b**) among the study groups.

Results are presented as mean ± standard deviation

*: Statistically significant compared to group C's corresponding value (P<0.05).

^: Statistically significant compared to subgroup Dc's corresponding value (P<0.05).

#: Statistically significant compared to subgroup Dl's corresponding value (P<0.05).

3.2. **Gastric COX-2 enzyme activity:** Figure (2) showed a highly statistically significant ($p < 0.01$) increase in COX-2 enzyme activity in the Dc (23.97 ± 2.46) and Dl (16.21 ± 1.07) rats and a statistically insignificantly ($p > 0.05$) decreased activity in the Df (10.67 ± 1.53) rats compared to the C (12.06 ± 1.17) rats. However, COX-2 enzyme activity was statistically significantly ($p < 0.01$)

decreased in Dl and Df rats compared to the Dc rats. Interestingly, the Df subgroup achieved a highly statistically significant (p < 0.01) protective effect against the elevated COX -2 enzyme activity compared to the Dl subgroup.
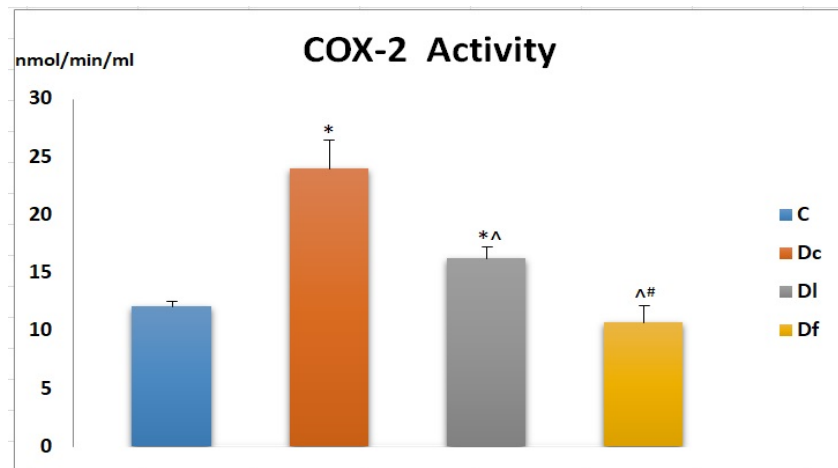

Figure (2): Cox-2 enzyme activity among the study groups.

Results are presented as mean ± standard deviation

*: Statistically significant compared to group C's corresponding value (P<0.05).

^: Statistically significant compared to subgroup Dc's corresponding value (P<0.05).

#: Statistically significant compared to subgroup Dl's corresponding value (P<0.05).

3.3. **Gastric homogenate gene expression of eNOS and GSH:** as shown in Figure (3-a and -b), the genes expression of eNOS and GSH in gastric homogenate was highly statistically significantly (p < 0.01) lower in Dc (0.14 ± 0.04 and 16.08 ± 3.06, respectively), statistically insignificantly (p > 0.05) higher in Dl (0.34 ± 0.07 and 33.97 ± 4.23, respectively), and highly statistically significantly (p < 0.01) elevated in Df (0.62 ± 0.05 and 45.53 ± 5.81, respectively) rats compared to C (0.33 ± 0.05 and 32.69 ± 4.99, respectively) rats. Nevertheless, the genes expressions were highly statistically significant (p < 0.01) increased in the Dl and Df subgroups compared to the Dc and Df subgroup compared to the Dl subgroup.
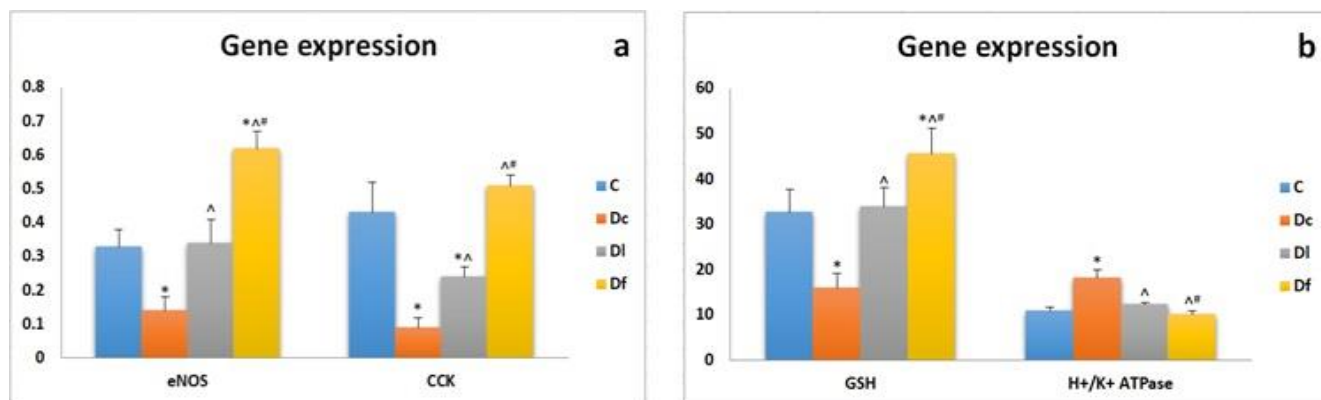

Figure (3): eNOS and CCK (**a**) and GSH and H^+/K^+ ATPase (**b**) genes expression among the study groups.

Results are presented as mean ± standard deviation

[*]: Statistically significant compared to group C's corresponding value (P<0.05).

[^]: Statistically significant compared to subgroup Dc's corresponding value (P<0.05).

[#]: Statistically significant compared to subgroup Dl's corresponding value (P<0.05).

3.4. **Gastric homogenate gene expression of CCK:** Figure (3-a) demonstrates that the gene expression of CCK in gastric homogenate was highly statistically significantly ($p < 0.01$) lower in Dc (0.09 ± 0.03), higher in Dl (0.24 ± 0.03), and statistically insignificantly ($p > 0.05$) higher in Df (0.51 ± 0.03) rats compared to C rats. Yet, the genes expressions were highly statistically significantly ($p < 0.01$) elevated in the Dl and Df subgroups compared to the Dc subgroup, and in the Df subgroup compared to the Dl subgroup.

3.5. **Gastric homogenate gene expression of H^+/K^+-ATPase:** From figure (3-b), we can notice in gastric homogenate H^+/K^+-ATPase gene expression a highly statistically significantly ($p < 0.01$) elevation in Dc (18.15 ± 1.86), statistically insignificantly ($p > 0.05$) elevation in Dl (12.37 ± 0.42), and statistically insignificantly ($p > 0.05$) drop in Df (10.14 ± 0.85) rats compared to C (10.87 ± 0.94) rats was. Nevertheless, the genes expressions were highly statistically significantly ($p < 0.01$) reduced in the Dl and Df subgroups compared to the Dc subgroup and the Df subgroup compared to the Dl subgroup.

3.6. **Histology and immunohistochemistry**

3.6.1. **H & E and Masson's Trichrome stains:** Histological examination of the gastric mucosa of group "C" rats showed disrupted gastric mucosa with blood vessels and leukocytes infiltrating the lamina propria (Fig. 4A) with less amount of collagen (Fig. 5A). In the subgroup "Dc", gastric mucosal was severely destroyed with loss and sloughing of gastric glands accompanied by significant infiltration with leukocytes in the lamina propria and submucosa (Fig. 4B). There was also an accumulation of excessive collagen fibers deposited in the peri-ulcer area in the gastric tissue with an increase in the thickness of the submucosa (Fig. 5B). Both groups "Dl" and "Df" showed mild disruption of the mucosa, regular gastric glands and normal submucosa (Fig. 4C and 4D), with less thickness of submucosa and decreased amount of collagen (Fig. 5C and 5D).

3.6.2. **Immunohistochemical staining:** Gastric wall of the fundus of the stomach of group "C" showed less dense COX-2 expression (Fig 6A). Sections of group "Dc" showed moderate COX-2 reactivity concentrated mainly at the peri-ulceration area (Fig 6B). COX-2 expression was less intense in the gastric wall of groups "Dl" and "Df" in

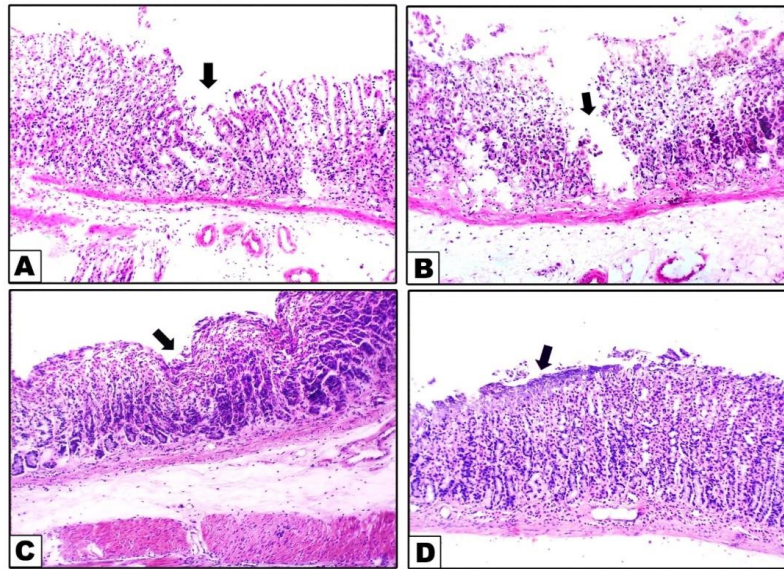comparison to the other groups (Fig. 6C   &   6D).

Figure (4): Sections of the stomach wall; A) group C (control) showing disentigrety and erosion of the gastric mucosa (black arrow), B) group Dc (diabetic control) showing sever destruction of gastric mucosa with loss and sloughing of gastric glands (black arrow), C) group Dl (leptin fed) showing mild disruption of gastric mucosa (black arrows), and D) Sections of group Df (PUFA-fed) showing mild disruption of gastric mucosa (black arrows). (Hematoxylin and eosin X 100)
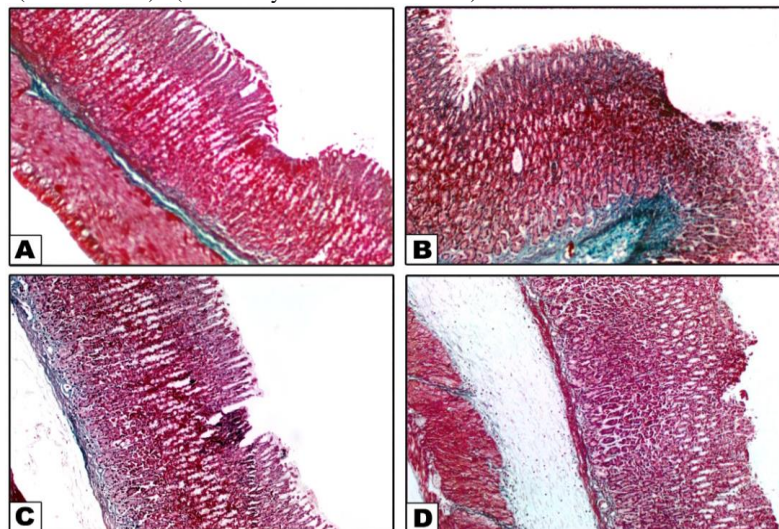

Figure (5): Sections of the wall of stomach; A) group C (control) showing moderate amount of collagen, B) group Dc (diabetic control) showing accumulation of excessive amount of collagen fibers, C) groups Dl (leptin fed) showing mild amount of collagen, and D) group Df (PUFA fed) showing mild amount of collagen fibers deposition.   (Masson Trichrome X 100)
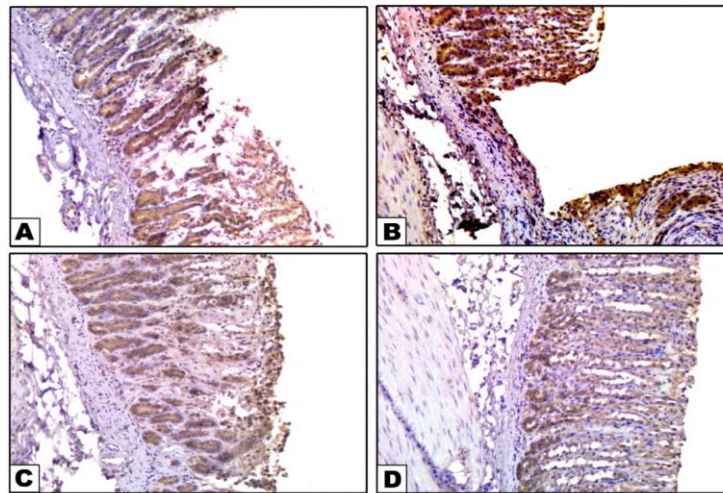
Figure (6): Immunohistochemically stained sections of the wall of stomach: A) group C (control) showing moderate COX-2 immunoreactivity, B) group Dc (diabetic control) showing severe COX-2 immunoreactivity, C) group Dl (leptin fed) showing mild COX-2 immunoreactivity, and D) group Df (PUFA fed) showing mild COX-2 immunoreactivity. (COX-2 immunstaining X 100)

4. Discussion

NSAIDs are group of drugs prescribed in conditions associated with pain, inflammation, and elevated body temperature such as rheumatoid arthritis, pyrexia, headache, migraine, acute gout, etc. Prolonged administration, inappropriate intake or even overdosage sometimes causes severe gastric ulcer and gastroduodenal disorders [30]. People with diabetes mellitus (DM) are at significant risk of developing numerous complications, including increased oxidative stress, chronic hyperglycemia, and decreased antioxidant enzymatic capacity [19]. They are also at increased risk of developing disorders related to the GIT and are more liable to ulceration.

In the current work, the gastric ulcer score, gastric COX-2 enzyme activity and gastric homogenate H^+/K^+-ATPase gene expression was highly statistically significantly increased in association with DM. In contrast, the percentage ulcer protection and gastric homogenate expression of eNOS, CCK and GSH genes were highly statistically significantly decreased with DM development. Our findings were supported by previously published results [31, 32]. The development of endothelial dysfunction impairs gastric blood flow in diabetic animals due to dysregulation of the eNOS/NO pathway [33]. This is usually associated with decreased oxygen delivery and multiple pro-inflammatory signalling pathways activation with excess roduction of pro-inflammatory cytokines and ROS. The result is oxidative tissue damage and ulcer formation [34]. On the other hand, the decreased glutathione (GSH) and increased COX-2 in diabetic animals is accompanied by prostaglandins (PG)-E_2 and -I_2 release [35]. The increased gastric acid and decreased CCK secretion will promote gastric ulcers [36, 37]. Notably, PG-E_2 overexpression suppresses gastric acid secretion and stimulates HCO_3^- secretion, which protects from the development of gastric ulcers [8].

Exogenously-given leptin acts as a trophic factor in the GIT via stimulating epithelial cell proliferation [38]. Leptin showed a stimulatory effect on intestinal mucosal morphometry development, epithelial cells proliferation, enzymatic activity in enterocytes brush border,

and in nutrient absorption [**39**]. Leptin receptor gene and protein expression were upregulated in the residual small inrtstinal mucosa following massive resection. This was associated with reactive increase in villus and crypt growth. Exogenous leptin also enhances all these effects [**40**]. Leptin-deficient ob/ob mice showed reduced cellular proliferation with elevated intestinal cells apoptosis following massive small bowel resection [**41**]. The leptin-induced satiety and the pancreatic secretions stimulation is blocked by a CCK-1-R antagonists [**42**]. The intestinal secretin tumor cells (STC)-1 secreting CCK have leptin receptors. In vivo duodenal leptin delivery increases serum CCK concentration. Feeding decreases gastric leptin and increases duodenum leptin even in leptin receptor-deficient mice; without affecting serum CCK levels [**43**]. The CCK-induced leptin release from gastric glands suggests a positive feedback loop between them [**44**].

Ω-3 PUFAs attenuate the inflammatory response through minimizing tissue injury without suppressing other inflammatory components necessary for subsequent wound healing [**45**]. Newly-discovered ω-3 PUFAs-derived lipid mediators synthesized during the later stages of inflammation, such as resolvins and protectins, promote inflammation resolution. They enhance apoptotic neutrophils phagocytosis by macrophage with subsequent macrophages migration to local lymph nodes [**46, 47**]. Resolvins improve inflammation resolution and microbial clearance in experimentally-induced critical illness [**48**]. Resolvins D_2 reduce leukocytes translocations to the sites of inflammation [**49**]. This reduces inflammatory infiltration, initiating the reparative stage of healing. Finally, fish oil supplementation is belived to prevent hypotension and improve oxygenation in critical illnesses [**50**], thus facilitating the healing of ulcers through maintaining cutaneous blood flow and oxygen supply and.

Ω-3 PUFA had proved its cytoprotective action by its antioxidant mechanism [**51**]. The beneficial effect of EPA- and DHA-rich fish might be due to the cell membrane AA displacement of phospholipid with preferential formation of less pro-inflammatory PGs (such as PG-E$_3$, -F$_{3\alpha}$, TX-A$_3$), and LTs (such as LT-B$_5$, LT-C$_5$, and -D$_5$) [**49**].

Conclusion

We can conclude from this study that both leptin and ω-3 PUFA have potent antacid, anti-inflammatory, and antioxidant effects through different mechanisms, guarding against the development of indomethacin-induced gastric ulcers in streptozotocin-induced type-II diabetes mellitus in male albino rats. In general, the protective effect of ω-3 PUFA was more potent than that of leptin. We can anticipate that the concomitant use of both of them should have a much more powerful synergetic anti-ulcer impact.

Funding

Conflicts of Interest

The authors contributing to this study have no conflict of interest. There was no external source of funding pertinent to this work

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