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A Comparative Biochemical and Histological Study of the Potential Protective Effects of Omega-3 and Vinegar on Stress-Induced Gastric Ulcers on Diabetic Rats

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Research Article

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ABSTRACT

Background: Diabetic patients are at high risk for variable complications and are more likely to develop gastrointestinal disorders, including gastric ulcers. In addition to the antidiabetic, anti-inflammatory, and antioxidant effects of omega-3 and vinegar, they can help in tissue healing.

Objective: We aimed to compare the effects of omega-3 and vinegar on gastric ulcers as one of the diabetic complications.

Methods: 24 rats were divided into negative control (n=6) and Streptozotocin-induced diabetes (n=18) groups. The latter group was further subdivided into three equal subgroups: diabetic control, diabetic+omega-3, and diabetic+vinegar. By the end of the experiment, ulcers were induced by water immersion/restraint. Gastric mucosa's gross appearance, histopathological parameters, and biochemical reactions were assessed.

Results: Consumption of omega-3 and vinegar significantly improved the ulcer index, percentage of ulcer protection, gene expression of gastric GSH, CCK, and e-NOS, and significantly decreased the gene expression of H^+/K^+-ATPase and COX-2 enzyme activity. The current study found that omega-3 has more potent antacid, anti-

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inflammatory, and antioxidant effects than vinegar on stress-induced gastric ulcers in diabetic rats.

Conclusion: We anticipated that combined use of both might have a synergetic anti-ulcer impact, supported by previously published studies.

INTRODUCTION

Diabetes Mellitus (DM) is a common metabolic disease characterized by chronic hyperglycemia and associated with increased production of Reactive Oxygen Species (ROS) and decreased antioxidant enzymes [1]. ROS are a naturally occurring byproduct of cellular metabolism. ROS levels in the low to moderate range are beneficial to a variety of physiological processes, including wound healing and tissue repair. Disproportionate ROS production, on the other hand, disrupts bodily homeostasis and causes oxidative tissue damage [2]. Diabetic patients are at higher risk for various macrovascular and microvascular dysfunctional complications, including neuropathy (somatic or autonomic), nephropathy, cardiovascular disorders, and gastrointestinal tract disorders [3]. In addition, diabetics are more likely to develop acute gastritis and/or peptic ulcers due to increased susceptibility of the gastric mucosa to various ulcerogens including stress [4]. In addition, diabetes increases the susceptibility to gastroparesis, which in turn increases the risk of gastric ulcer due to prolonged exposure of the poorly protected gastric mucosa to gastric acid [5].

Stress is the body's natural defense against intense psychic or physical change. However, a strong association has been found between prolonged exposure to psycho-physical stress and the development of gastric ulcers [6]. Stress-induced gastric ulcers have been extensively studied in both human and animal models. The pathogenesis of gastric ulcers is variable and can be caused by inflammation, oxidative stress, increased gastric acid secretion (HCL), decreased gastric prostaglandin production, or inhibition of gastric mucosal proliferation [7]. The proton pump hydrogen-potassium adenosine triphosphate (H^+/K^+-ATPase), which regulates the exchange of cytoplasmic H+ and extracellular K^+, controls gastric acid production. Where H+ was released to react with Cl^- in the gastric lumen to form HCL. Inhibition of H^+/K^+ ATPase activity, on the other hand, reduces gastric HCL production and protects against ulcer formation [8]. Prostaglandin (PG) production is influenced by cyclooxygenase (COX)-1 and COX-2, which are expressed in different types of gastric mucosal cells. PG-E2 and PG-I2 are usually the more prominent subtypes in the gastric mucosa, and their overexpression reduces or inhibits gastric HCL secretion [9,10].

Omega-3, also known as long-chain Polyunsaturated Fatty Acids (PUFA), is found in a variety of foods, including fish, seafood, green leafy plants, and nuts [11]. PUFA, particularly Eicosapentanoic Acid (EPA) and Docosahexanoic Acid (DHA), have been shown to have numerous health benefits and help treat a variety of pathological conditions. EPA is converted into prostaglandins, thromboxanes, and leukotrienes, all of which have potent anti-inflammatory properties [12]. In addition, PUFA has an antidiabetic effect by reducing inflammation, blood glucose levels, insulin resistance, and improving insulin sensitivity [13,14]. Omedga-3 may also have a protective effect on the stomach against injury and ulceration [15].

Vinegar is made by fermenting starchy or sugary grains or fruits and has always been used as a food preservative. Vinegar is an aqueous solution containing up to 5% acetic acid in water, as well as salts and some fermentation products [16]. Vinegar is believed to have a variety of health benefits, including antibacterial, anti-inflammatory, and antioxidant properties, the

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ability to regulate blood sugar and fat metabolism, and the ability to speed tissue healing. In addition, vinegar may lower fasting blood glucose levels in diabetics and prediabetics [17,18].

To our knowledge, there is little evidence on the beneficial role of omega-3 or vinegar in the prevention of gastric ulcers. As a result, we aimed to investigate the potential protective effects of omega-3 versus vinegar in the prevention of stress-induced gastric ulcers in Streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Experimental animals

Twenty-four male albino rats, weighing 160 g ± 15 g, were obtained from the Animal House of Kasr Al-Aini Faculty of Medicine, Cairo University, Egypt. The rats were observed for seven days for adaptation before the start of the experiment and rats showing signs of disease or infection were excluded. The animals were then housed in wire mesh cages (50 cm × 20 cm × 20 cm, three rats/cage) with well-ventilated covers at room temperature (25℃ ± 5℃) with 12 h alternating light/dark cycle. Rats had free access to water and ad-lib food. The experimental protocols of the study were approved by Cairo University's Institutional Animal Care and Use Committee (CU-IACUC), with approval number "CU/III/F/64/17". The animals were then divided into the following groups:

- Group I (negative control, n=6): rats were fed with normal rodent food ad-lib for six weeks.
- Group II (diabetic control,n=18): animals were fed a short-term high-fat diet (HFD), (caloric content: carbohydrate 26.0%, protein 15.2%, fat 58.8%) for 2 weeks. Subsequently, two low doses of 30 mg/kg Streptozotocin (STZ) in 0.01 mol/l citrate buffer (STZ; Sigma, St. Louis, USA) were injected intraperitoneally (IP) 24 hours apart. DM was confirmed by measuring fasting blood glucose (FBG) and insulin. Only rats with FBG value ≥ 200 mg/dl after five days measured by One Touch Ultra glucose meter, Life Scan, Milpitas, CA USA, were included in the study as diabetic animals.Thereafter, the animals were further divided into three equal subgroups (n × 6 each) as follows:

- Subgroup II a (diabetic control): rats were fed a normal rodent diet for six weeks.
- Subgroup II b (PUFA): rats were fed a normal rodent diet fortified with 100 g/kg omega-3 PUFA for six weeks [19].
- Subgroup II c (Vinegar): Rats were fed a normal rodent diet enriched with 15% natural vinegar (5% acetic acid) for six weeks 18

Design of stress-induced gastric ulcer: rats were immobilized in a stress cage and then immersed in a water bath at 23℃ ± 0.2℃ at the level of the xiphoid for 4 hours [20]. Rats were then removed from the cage and sacrificed using the CO_2 euthanasia method [21].

RESULTS

The stomach of all rats was carefully removed and cut open along major curvatures. The following parameters were measured:

Ulcer score: ulcers were counted using a hand magnifying lens (10X), and the severity of each lesion was scored on a scale of 1-3 as follows: '1' if the ulcer was less than 1 mm (pinpoint), '2' if it was 1 mm-2 mm, and '3' for ulcers larger than 2 mm [22].

- Percentage of ulcer protection [23]: Ulcer protection (%)=(Uc-Ut/Uc) × 100

Where: Uc=ulcer index of control group.

Ut=ulcer index of test group.

*Ulcer index was calculated by dividing the ulcer score by a factor of 10.

- Biochemical assays:

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- COX-2 activity: COX-2 peroxidase activity was measured colorimetrically as described previously [24].
- Expression of endothelial nitric oxide synthase (eNOS), CCK, GSH and H^+/K^+-ATPase genes by real-time RT-PCR: Total RNA was extracted from the gastric tissue homogenate using total RNA Isolation Kit (Invitrogen, CA). Single-strand cDNA was synthesized using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). Quantitative RT-PCR was performed using TaqMan Fluorescein one step method with specific primers as shown in Table 1 [25].

Table 1. Oligonucleotide primers used for PCR analysis.

Gene	Primer used
eNOS	F* 5′-TCCGGAAGGCGTTTGATC-3′ R* 5′-GCCAAATGTGCTGGTCACC-3′
CCK	F* 5′ GCGATTTGCAAACCCTTACAG-3′ R* 5′ CACCTTCAAAGCATGGGATTTT-3`
GSH	F* 5` GGAACGACAACCAGGGACTA 3` R* 5` TCCCTGGACGGACATACTTC 3`
H^+/K^+-ATPase	F* 5` CTTTGCCATCCAGGCTAGTGA 3` R* 5` CTTTGCCATCCAGGCTAGTGA 3`

- **Histopathological examination:** after quantification of ulcer score, stomachs were fixed in 10% neutral buffered formalin, processed and embedded in paraffin. Sections 4 µm thick were cut and then stained with hematoxylin and eosin (H&E, Scytek Laboratories SDP Hematoxylin-Eosin Stain Kit, catalog no. NC0510871) according to the manufacturer's protocols.
- **Immunohistochemical staining:** formalin-fixed, paraffin-embedded sections were cut at 4 µm. Sections were deparaffenized in xylene, rehydrated, and stained with the anti-COX2 antibody, [EP8588]-(ab169782)-Abcam, according to the manufacturer's protocol. Positive immunoreactions appeared as brown staining. Negative controls were performed by omitting the primary antibody, resulting in negative immunoreactivity. To ensure reproducibility, five different animals from each group were tested and their sections were processed simultaneously. The sections were examined and photographed. Immunohistochemical results were calculated semi-quantitatively using a scale of 0 to 3 (0 absent, 1 mild [10% of positive cells], 2 moderate [10% to 25%], and 3 severe [25%]) [26].
- **Statistical analysis:** Data were coded and entered using the Windows® statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Data were summarized using mean and standard deviation for quantitative variables. ANOVA with multiple comparisons post hoc test was used to analyze statistically significant differences between the means of data from different groups. P values less than 0.05 were considered statistically significant [27].
- **Score of gastric ulcers and percentage of ulcer protection:** As shown in Table 2 and Figure 1, the ulcer score was significantly higher ($p \leq 0.01$) in diabetic control rats (12.17 ± 0.68) compared to normal rats (9 ± 0.58), with a 35% reduction in ulcer protection. This deterioration was significantly reduced ($p \leq 0.01$) in both PUFA-fed (6.83 ± 0.89) and

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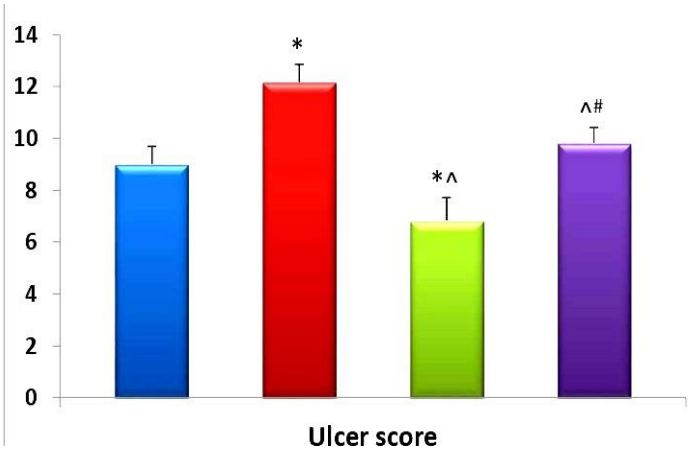
vinegar-fed (9.83 ± 0.69) subgroups, resulting in a higher overall percentage of ulcer protection (24.33% and -9.22%, respectively). Surprisingly, PUFA-fed animals had a significantly greater ($p \leq 0.01$) protective effect than vinegar-fed animals.

Table 2. Different biochemical and histopathological parameters among the study groups.

	I	II		
		a	b	c
Ulcer score	9 ± 0.58	12.17 ± 0.68*	6.81 ± 0.89*^	9.83 ± 0.69^#
%protection		-35.22	24.33	-9.22
COX-2 (nmol/min/ml)	11.65 ± 1.05	24.08 ± 2.2*	10.83 ± 1.2^	15.8 ± 1.32*^#
eNOS	0.28 ± 0.04	0.12 ± 0.04*	0.68 ± 0.03*^	0.29 ± 0.06^#
CCK	0.39 ±0.07	0.1 ± 0.02*	0.44 ± 0.02^	0.2 ± 0.02*^#
GSH	30.73 ± 5.34	15.55 ± 2.81*	41.23 ± 5.43 *^	29.72 ± 3.8^#
H^+/K^+ ATPase	11.17 ± 0.72	17.82 ± 2.07*	10.38 ± 0.67 ^	12.68 ± 0.58^#

Note: (*):Statistically significant compared to corresponding value in subgroup I (P<0.05); (^): Statistically significant compared to corresponding value in subgroup IIa (P<0.05); (#):Statistically significant compared to corresponding value in subgroup IIb (P<0.05).

Figure 1. Comparison of the ulcer score among the study groups. **Note:** Groups: I (negative control), IIa (diabetic control), IIb (ω-3 fed), and IIc (vinegar fed). Values are presented as mean ± SD. Values are statistically significant (P<0.05) as compared to their corresponding values in: (*): group I, (^): group IIa, and (#): group IIb.



- **Gastric COX-2 enzyme activity:** Table 2 and Figure 2 showed a significant increase ($p \leq 0.01$) in COX-2 enzyme activity in the diabetic control subgroup (24.08 ± 2.2) compared to the normal group (11.65 ± 1.05). On the other

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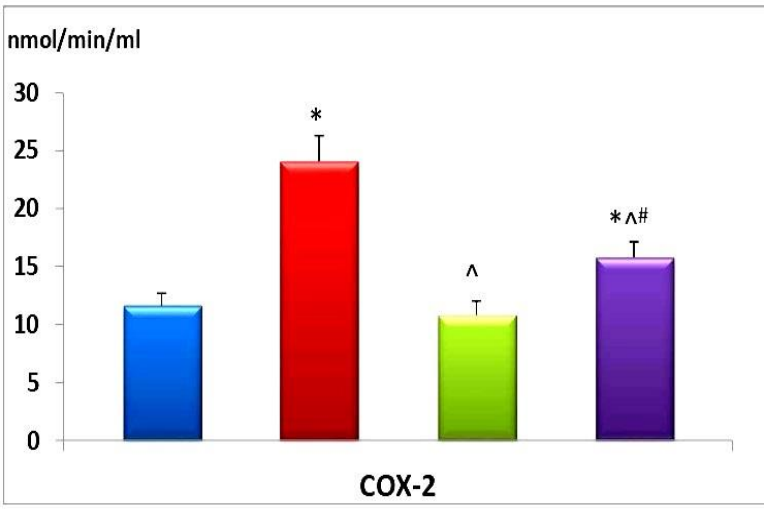
hand, COX-2 enzyme activity significantly ($p \leq 0.01$) decreased in both PUFA-fed (10.83 ± 1.2) and vinegar-fed (15.8 ± 1.32) subgroups compared to diabetic control group. Interestingly, PUFA-supplemented diet achieved a strong protective effect against the increased COX-2 enzyme activity.

Figure 2. Comparison of COX-2 enzyme activity among the study groups. **Note:** Groups: I (negative control), IIa (diabetic control), IIb (ω-3 fed), and IIc (vinegar fed). Values are presented as mean ± SD. Values are statistically significant ($P<0.05$) as compared to their corresponding values in: (*): group I, (^): group IIa, and (#): group IIb.



- **The eNOS, CCK and GSH gene expression of the gastric homogenate:** as shown in Table 2 the gene expression levels of eNOS (0.12 ± 0.04), CCK (0.1 ± 0.02) and GSH (15.55 ± 2.81) were significantly lower ($p \leq 0.01$) in the diabetic control subgroup compared to the normal control animals (00.28 ± 0.04, 0.39 ± 0.07 and 30.73 ± 5.34, respectively). On the other hand, the expression of these genes was significantly increased ($p \leq 0.01$) in the PUFA-fed subgroup (0.68 ± 0.03, 0.44 ± 0.02 and 41.23 ± 5.43, respectively) and the vinegar-fed subgroup (0.29 ± 0.06, 0.2 ± 0.02 and 29.72 ± 3.8, respectively). However, the improvements in gene expression were greater in the PUFA-fed group than in the vinegar-fed group.
- **H^+/K^+-ATPase gene expression of gastric homogenate:** Table 2 showed a significant increase in H^+/K^+-ATPase gene expression ($p \leq 0.01$) in diabetic control rats (17.82 ± 2.07) compared to the normal group (11.17 ± 0.72). However, this level of gene expression was significantly lower ($p \leq 0.01$) in PUFA-fed (10.38 ± 0.67) and vinegar-fed (12.68 ± 0.58) rats compared to normal control rats ($p=0.71$ and $p=0.21$, respectively). Surprisingly, the improvement in gastric expression of the H^+/K^+ ATPase gene was greater ($p \leq 0.05$) in PUFA-fed rats than in vinegar-fed rats.

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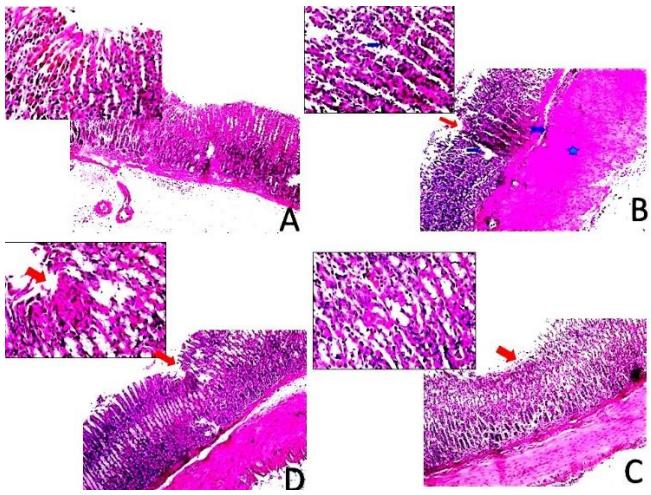
Histology and immunohistochemistry

- **Hematoxylin and eosin stain:** Histological examination of the gastric mucosa of normal rats showed a normal pattern of surface epithelium and gastric gland. The lamina propria is broad and infiltrated by blood vessels and leukocytes. In contrast, in the diabetic control rats, the epithelium of the gastric mucosa was severely disrupted, with extensive erosion of the mucosa, detachment of the gastric glands, and sloughed cells of the lumen (red arrow). There was also severe infiltration of inflammatory cells (blue arrow) and a thickened muscle layer (*). In PUFA-fed and vinegar-fed rats, gastric tissue sections improved morphologically. Mild mucosal disturbances, regular gastric glands, and normal submucosa were observed, along with mild infiltration of inflammatory cells.

Figure 3. Hematoxylin and eosin-stained sections (100x and 200x) of **A)** group I (negative control) revealed disruption of surface epithelium and gastric gland. The lamina propria is wide and infiltrated with many blood vessels and inflammatory cells, **B)** group iia (diabetic control) showed severe disruption of gastric mucosal epithelium, extensive mucosal erosions in the glandular part of the stomach, sloughing of gastric glands, and exfoliated cells appearing in the lumen (red arrow). Leucocyte infiltration was detected (blue arrow) with a thickness of muscle layer (astrx), **C)** group iib (PUFA-fed) showed mild disruption of the mucosa, regular gastric glands and normal submucosa with mild inflammatory infiltration, and **D)** group iic (vinegar-Fed) showed disruption of surface epithelium and gastric gland. The lamina propria is infiltrated with inflammatory cells.



- **Immunohistochemical staining:** Compared with the normal control group, the gastric tissue of the diabetic control group showed strong COX-2 reactivity, which was mainly visible at the base of the gastric glands and in the

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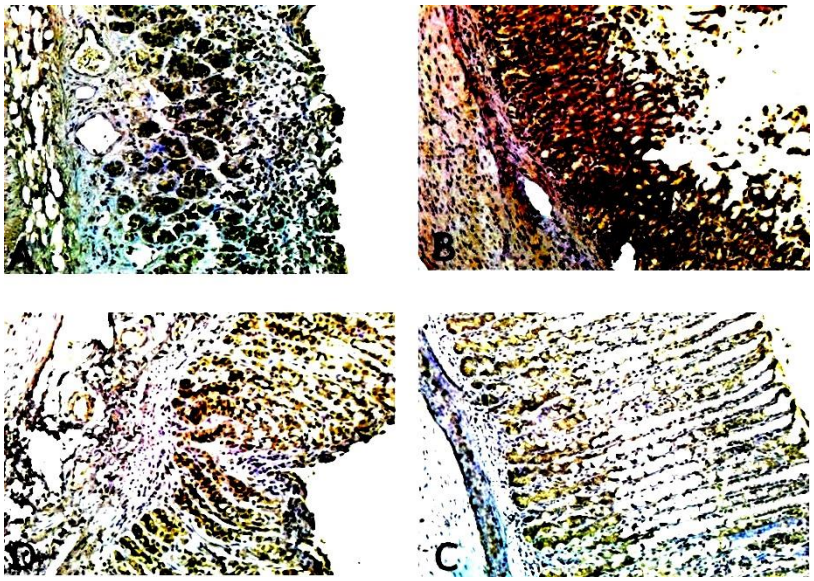
inflammatory cells of the submucosal layer. COX-2 expression in gastric tissues was lower in the PUFA-fed subgroup compared with the vinegar-fed subgroup (Figures 2-4).

Figure 4. Immunohistochemical staining for anti-COX-2 antibodies (200X) showed: **A)** group I (negative control) with moderate reaction, **B)** group iia (diabetic control) with a strong reaction, **C)** group iib (PUFA-fed) with a mild reaction, and **D)** group iic (vinegar-Fed) with moderate reaction.



DISCUSSION

Diabetics are at high risk of developing various complications related to chronic hyperglycemia, increased oxidative stress, and decreased antioxidant enzymes [1]. Diabetics also have a higher risk of developing gastrointestinal disorders and are more prone to ulceration. In addition, stress has been linked to the development of gastric ulcers [28]. In combination, stress and diabetes may accelerate the development of ulcers. On the other hand, PUFA and vinegar have been attributed with various biological benefits, such as antidiabetic, anti-inflammatory, antioxidant, and tissue repair stimulating properties. Currently, diabetes were induced after STZ injection and gastric ulcers after stress, as previously distinguished [20].

In the present study, gastric ulcer score, COX-2 enzyme activity and H^+/K^+-ATPase gene expression were significantly increased ($p \leq 0.01$), while ulcer protection percentage, eNOS, CCK and GSH gene expressions were significantly decreased in the diabetic group compared to normal rats ($p \leq 0.01$). Our findings were supported by previously published result [19,29,30] Stress causes endothelial dysfunction with impaired gastric blood flow in diabetic animals due to dysregulation of the eNOS/NO

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pathway [31]. This in turn leads to a decrease in oxygen delivery along with activation of multiple pro-inflammatory signaling pathways. When these pathways are disrupted, pro-inflammatory cytokines and Reactive Oxygen Species (ROS) are overproduced, leading to oxidative tissue damage and ulcer formation [32]. Moreover, NOS is normally expressed in the gastrointestinal mucosa and helps regulate gastric mucosal blood flow, epithelial secretion, and barrier function to maintain normal mucosal functions and integrity. Overexpression of NO, on the other hand, has a deleterious effect on the GIT mucosa, as shown in patients with chronic ulcerative colitis and peptic ulcers [2]. In contrast, in the diabetic animals, decreased glutathione (GSH) and increased COX-2 lead to the release of prostaglandin (PG)-E2 and PG-I2 [33] Also, decreased CCK secretion, which is important for the gastric mucosal integrity and protection against ulceration, and increased gastric acid secretion will promote gastric ulcers [34,35]. Surprisingly, overexpression of PG-E2 stimulates bicarbonate secretion while suppressing gastric acid secretion, which protects against gastric ulceration [8].

In the current study, PUFA were found to be effective in reducing stress-induced ulceration in diabetic rats. This result was achieved through a variety of mechanisms, including anti-inflammatory, antioxidant, and antidiabetic pathways. As previously mentioned, PUFA have an antidiabetic effect by reducing inflammation, lowering blood glucose levels, decreasing insulin resistance, and increasing insulin sensitivity [13,14]. PUFA, especially Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA), have been associated with the formation of anti-inflammatory PG-D2, PG-E1, PG-Is, and leukotrienes (LTs), such as LTB5, LTC5, and LTD5. In addition, PUFA mediators such as Resolving-D2, Protectins, and Protein-D reduce trafficking of leukocytes to inflammatory sites as well as the expression of CD26L and CD18, which inhibits inflammation and stimulates the tissue repair pathway [36] In addition, PUFAs improve mucosal microcirculation and stimulate angiogenesis at the ulcer margin, which increases tissue perfusion and gastric content of mucin and GSH [37,38]. PUFAs, also stimulates the secretion of CCK and inhibits the release of gastric acid and gastrin. Taken together, these pathways maintain blood flow and oxygen supply to the gastric mucosa and facilitate ulcer healing. It has also been reported that increased CCK secretion or exogenous administration accelerates ulcer healing [39].

The H^+/K^+-ATPases are divided into two types: gastric and non-gastric. The gastric H^+/K^+-ATPase is mainly located in the parietal cells of the stomach, where it is associated with H^+ secretion, K^+ absorption, and K^+ recirculation. Non-gastric H^+/K-ATPase is involved in acid-base or K^+ and Na^+ homeostasis and is found in a number of tissues including the colon, kidney, skin, placenta and prostate. Gastric H^+/K^+-ATPase is the main target for the treatment of peptic ulcers. Proton pump inhibitors can suppress gastric or non-gastric H^+/K^+-ATPase [40]. In this study, we hypothesized that PUFA acts as a proton pump inhibitor to protect diabetic rats with stress-induced gastric ulcers from gastric activation of the H^+-K^+pump. This effect could be attributed

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to either a down regulation of Na^+/K^+-ATPase gene expression or a decrease in its activity, since high PUFA concentrations can alter membrane properties and inhibit Na^+-K^+ pump activity [41].

Vinegar-fed animals showed a significant reduction in the mean values of gastric H^+/K^+-ATPase gene expression and COX2 enzyme activity, explaining the antacid and anti-inflammatory effects of vinegar. Due to its diverse chemical composition, which includes histidine, acetic acid, and phenol, vinegar is considered a potent anti-inflammatory and antioxidant against ROS [42]. Phenolic compounds act as nucleophiles (i.e. electron donors), scavenging free radicals and converting them into more stable products [43]. It was reported that vinegar can improve antioxidant capacities and reduce oxidative damage in human and in animals [18]. Synthetic vinegar has been shown to increase SOD, CAT and glutathione peroxidase activities and reduce lipid peroxidation levels [44]. In addition, vinegar, particularly black vinegar, can reduce or suppress COX2 enzyme activity and accelerate wound healing [45] On the other hand, vinegar, particularly Bamboo vinegar, can affect eNOS in a dose dependent manner [46]. Moreover, organic acids derived from vinegar fermentation have antimicrobial properties, help regulate blood glucose levels, and contribute to the regulation of lipid metabolism [47]. Also, vinegar has been reported to have vasodilatory effects and promote tissue healing [17,48]. Suggested that vinegar acetic acid can activate e-NOS by phosphorylation of the Ser1177 residue in a dosage-dependent biphasic manner, which in turn leads to flow-mediated vasodilatation in humans. In addition, Daugirdas and Nawab [49]. reported that acetate induced relaxation of the vascular smooth muscle. However, phenols caused intracellular acidification, which disrupted H^+/K^+-ATPase. It has been previously reported that phenols, such as those found in apple cider vinegar, inhibit H^+/K^+-ATPase and *H. pylori* growth in a structurally dependent manner [50].

CONCLUSION

Taken together, gastric mucosal integrity by increased CCK secretion, increased mucosal blood flow by e-NOS as well as inhibition of H^+/K^+-ATPase by phenolics of vinegar, can provide a good protection against stress induced ulcer in diabetic animals. The current study found that PUFA has more potent antacid, anti-inflammatory, and antioxidant effects than vinegar on stress-induced gastric ulcers in diabetic rats. We anticipated that combined use of both might have a synergetic anti-ulcer impact, supported by previously published studies.

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