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The protective role of exogenous Ghrelin versus its combination with Zingerone on experimentally induced gastric ischemic-reperfusion in adult male albino rats

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ABSTRACT

Ghrelin is a gut hormone has stimulatory properties on food intake, fat deposition and growth hormone release. Zingerone is a component of ginger with multiple pharmacological activities. They were established that have protective roles against oxidative stress actions. We planned this study to evaluate pretreatment exogenous Ghrelin alone and or accompanied with Zingerone on ischemia-reperfusion injury to gastric fundus wall. Fifty male albino rats were used and subdivided into control, ischemic-reperfusion, Ghrelin pretreated and Ghrelin Zingerone pretreated groups. Specimens from the stomach fundus were processed for histological, immunohistochemical study and gene expression using real time PCR. Morphometric and statistical analyses were also carried out in this research. In ischemic-reperfusion sections, there were deep erosion and distortion of the mucosa. Chief cells appeared with vacuolated cytoplasm and pyknotic nuclei. Congestion of blood vessels with extravasation and cellular infiltration was also noticed. There was a decrease in mucous secreted cells in PAS-stained sections. Sections from Ghrelin pretreated and Ghrelin Zingerone pretreated groups showed a great improvement. In addition, gastric tissues with the ischemia-reperfusion group showed a significant decrease in enos and nrf2 mRNA expression while there was a significant increase in HIF and VEGF, which is counteracted to Ghrelin pretreated and Ghrelin Zingerone pretreated groups. This study revealed the vital protective role of Ghrelin in concomitant with Zingerone than pretreated Ghrelin alone on attenuating the damage changes of fundus that occurred after experimentally induced gastric ischemia-reperfusion.

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Introduction

The gastric wall has a rich collateral blood supply, so some studies mentioned that its ischemia may be a rare condition (1). Its occurrence may due to obstruction of the arterial or venous blood flow as in thromboembolism or gastric volvulus. The non-obstructive causes may lead to a reduction of cardiac output and gastric ischemia as in congestive heart failure and side effects of digitalis and alpha-adrenergic agents (2). Ischemia-reperfusion injury (IRI) results from a prolonged ischemic insult followed by a restoration of blood perfusion. This can distress all oxygen-dependent cells relying on continuous blood supply and in that way damage occurs to aerobically metabolizing organs and tissues (3).

Ghrelin was firstly identified, purified and described by Kojim in 1999 from rat stomach. It is produced and secreted from the A-like cells found mainly in the oxyntic glands of the gastric fundus (4). Experimental and clinical studies mentioned that the Ghrelin-producing cells and Ghrelin mRNA expression are present in different tissues and organs as the kidney, large bowel, rectum, small bowel, thyroid, human placenta and pancreas (5).

Previous researchers mentioned that Ghrelin is now known to show a role not only in growth-hormone release but also in motivating gastric motility and food intake. Exogenous Ghrelin protects and exerts beneficial anti-inflammatory effects in colonic mucosa resulting in better intestinal mucosal healing and could be applied to ameliorate the outcomes in colorectal surgery (6). Bianchi et al. postulated that the administration of it in post-operative laparotomy reduces the peritoneal adhesions and fibrotic response so it has an anti-adhesion effect (7).

Natural products are main sources for the prevention and treatment of many gastric insults. Zingerone (ZO) (4-hydroxy-3 methoxyphenyl)-2-butanone) is a component of ginger (Zingiber

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officinale) that has different pharmacological roles. It has anti-inflammatory, anticancer, anti-diabetic, anti-diarrheal, antispasmodic and anti-thrombotic (8, 9). The activity of ZO has been shown in various experimental animal models of many diseases and its effect is equal to ascorbic acid (10, 11).

The present study was designed to investigate the influences of pretreated exogenous Ghrelin versus its combination with Zingerone on experimentally induced ischemia-reperfusion of the wall of the gastric fundus in a rat model.

Materials and methods

Zingerone: (CAS 122-48-5, Purity ≥96%, Molecular Weight 194.23 Da) was purchased in the form of powder from Sigma Aldrich trade company, Cairo, Egypt. Its chemical formula: Vanillylacetone (4-(HO) C6H3-3-(OCH3) CH2CH2COCH3.

Experimental animals:

Fifty adult male albino rats (200–250 g body weight) were purchased from the center of experimental animals, Faculty of Veterinary medicine, Zagazig University. They were housed in the Animal House of the Faculty of Medicine, Zagazig University under standard conditions (12 h light/dark cycle; 25±3¯C, 45–65% humidity) and had free access to standard rat feed and water ad libitum. All the animals were acclimatized to laboratory conditions for a week before the beginning of the experiment. All experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee accepted by the Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Experimental design:

The animals were randomly assigned to:

Group I (control group): Included twenty rats further subdivided equally into two subgroups:

Subgroup Ia: (sham-operated group): Each rat underwent laparotomy without inducing I/R injury (12).

Subgroup Ib: (positive control group): Zingerone was given in a dose of 50 mg/kg through a nasogastric tube for 7 days (13).

Group II (ischaemic reperfusion (IR) group): Included ten rats, underwent acute gastric ischemia for 30 minutes, then reperfusion for 24 hours (12).

Group III (pretreated group): Twenty rats were further subdivided equally into two subgroups:

Subgroup IIIa (Ghrelin pretreated group): Exogenous Ghrelin was given in a dose of (50 μg/kg) intraperitoneal (i.p) 30 min prior to the experiment (14).

Subgroup IIIb (Ghrelin and Zingerone pretreated group): Zingerone was administered by nasogasric tube for 7 days in a dose of 50 mg/kg (13). 30 min prior to the experiment each in conjugation with exogenous Ghrelin (50 μg/kg i.p.) (14).

Preparation of the gastric ischemia-reperfusion model:

The rats were anesthetized with Thiopental (50 mg/kg, i.p.) (Sigma-Aldrich Co., St Louis MO, USA) for general anesthesia (12)). The rats were placed on a heating blanket (37°C) to maintain their body temperature and then fixed on the operating table in a supine position. With a median incision through the linea alba (15). The celiac artery and its adjacent tissues were carefully separated. The celiac artery was clamped using a ligature for 30 min to induce ischemia, and the ligature was then removed to allow reperfusion for 24 hours (12, 16).

General observations of animals

During the experimental period, the clinical signs and general appearances were checked daily. Mortalities of the rats were recorded.

At the end of the experiment, rats of all groups were sacrificed with an intraperitoneal injection of 25 mg/kg sodium thiopental (17). Specimens from the stomach fundus from the four groups were removed and further subdivided into two portions. The first part was stored at - 80°C for RNA extraction. Samples from the second part were processed for histological examinations.

Real-time PCR analysis

Total RNA was isolated from frozen gastric tissue according to the RNA isolation kit (RNeasy Mini Kit, Qiagen) following the manufacturer's protocol. For the synthesis of complementary DNA (cDNA), the extracted RNA was reverse transcribed by (QIAGEN OneStep RT-PCR Kit), as recommended by the manufacturer. Expression levels of eNOS, VEGF, HIF and Nrf2 mRNA were determined by real time polymerase chain reaction using Mx3005P

(Stratagene, USA). Glyceraldehyde- 3- phosphate dehydrogenase (GAPDH) is used as an internal control. Primers were reported in Table 1.

The PCR was performed in 25 µL containing 12.5 µL QuantiFast SYBR Green (Qiagen) PCR Master Mix, 10 pmol of each primer (Invitrogen, USA) and 5 µL cDNA. For eNOS, amplification conditions were: 3min at 95°C, 30s at 60°C and 45s at 72°C for 35 cycles. Thermal cycling conditions for VEGF and HIF were as followed: denaturation at 94 °C for 1 min, annealing at 60 °C for 45 s and extension at 72 °C for 2 min, the number of cycles was 33. For Nrf2 and GAPDH PCR reactions included 15 min at 95°C, followed by 45 cycles at 94°Cfor 15 sec (denaturing), 55°C for 30 sec (annealing), and 72°C for 30 sec (extension). The relative expression was calculated from the $2^{-\Delta\Delta CT}$ formula.

Table 1. The primer (Invitrogen, USA) sequence for

Gene	Primer sequences
eNOS	forward: 5'-GGAGAAGATGCCAAGGCTGCTG-3', reverse: 5'-CTTCCAGTGTCCAGACGCACCA-3',
Nrf2	forward:5'-ATGGCCACACTTTTCTGGAC-3'; reverse: 5'-AGATGTCAAGCGGGTCACTT-3'
VEGF	forward: 5' GGTCCTCTGCCATTCT -3' reverse: 5' CTTGCCTTGCTGCTCTAC -3'
HIF	forward: 5' TCTGGACTCTCG CCTCTG - 3' reverse: 5' GCTGCCCTTCTGACTCTG - 3'
GAPDH	forward: 5' -GGGCAGCCCAGAACATCA 3', reverse: 5' -TGACCTTGCCCACAGCCT-3'

Histological study

For histological preparation, tissues were fixed in 10% neutral formalin for 24 hrs. The tissues were dehydrated, cleared, embedded in paraffin and sliced into 4 µm. Sections were de-waxed, rehydrated and stained with eosin and hematoxylin (H&E) and Periodic Acid and Schiff (PAS) stains procedure were used (18).

Immunohistochemical study

Dewaxed Paraffin tissue sections were processed for Immunohistochemical detection of tumor necrosis factor -α (TNF- α) (index for inflammation) and anti-ghrelin. These studies were carried out using the peroxidase-labeled Streptavidin- Biotin. The technique was carried out according to Ramos-Vara et al. (19). Paraffin sections (5 µm) were deparaffinized and rehydrated down to distilled water. The endogenous peroxidase activity was inhibited by 3% Hydrogen peroxide H_2O_2 in methanol for 10 min. after washing the sections with phosphate-buffered saline (PBS). A blocking solution was used for the nonspecific staining (1.5% normal goat serum in PBS). After that sections were incubated at 4°C overnight with the primary antibody.

Tumor necrosis factor -α (TNF-α)

The primary monoclonal antibody used was the mouse anti-TNF-α (Santa Cruz Biotechnology, Santa Cruz, California, USA) (1:300 with PBS). The cellular site of the reaction was cytoplasmic brown in color.

Anti-ghrelin

The expression of ghrelin was detected using a rabbit polyclonal antibody (sc.50297 Santa Cruz Biotechnology, CA, USA).

Secondary antibody (goat anti-mouse Ig G-horseradish peroxidase conjugate from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added in order of priority and then diaminobenzidine (DAB) staining and hematoxylin counterstaining were performed. The primary antibody was replaced with PBS as a negative control.

Morphometric analysis

Ten slides from 10 different specimens of each group were examined and from each slide, ten non-overlapping fields The mean area percentage of PAS-positive reaction, positive immunoreactivity for TNF-α and anti-ghrelin were measured using Leica Qwin 500 image analysis computer system (Leica Microsystems Ltd, Cambridge, UK) at the Pathology Department, Faculty of Medicine, Cairo University. They were calculated using the color detect menu and in relation to a standard measuring frame.

Ten slides from 10 different specimens of each group were examined and from each slide, ten non-overlapping fields using an objective lens at a magnification of ×400.

Statistical analysis

Data were analyzed using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc Scheffe's test was applied to compare differences among the groups. In each test, data was evident as the mean (M) value ± standard

deviation (SD) and differences were considered to be significant at $P < 0.01$. 153.

Results

Characterization of Gastric HIF-1α, VEGF, eNOS and Nrf2 mRNA expression by real-time PCR analysis in the different studied groups:

In the present study, an ischemic-reperfusion group revealed a significant decrease in eNOS and nrf2 mRNA expression, while there was a significant increase in HIF and VEGF which may be attributed to hypoxia stimulation. Interestingly, Ghrelin pretreated group was able to counteract the I/R-related effects. Moreover, in Ghrelin and Zingerone pretreated group, there were a significantly increased expression of HIF-1α, VEGF, eNOS and nrf2. We also revealed that Zingerone inhibits oxidative stress following acute ischemia/ reperfusion by increasing eNOS and Nrf2 mRNA expression significantly in addition to increasing HIF-1α and VEGF mRNA expression (Table 2).

Table 2. Gastric gene expression analysis of eNOS, Nrf2, VEGF, HIF

	Enos	Nrf2	VEGF	HIF
Control	1.15±0.12	1.02±0.09	1.2±0.11	0.98±0.15
Ischemia reperfusion	0.37±0.08[a]	0.42±0.12[a]	1.6±0.28[a]	1.2±0.19[a]
Ghrelin pretreated	0.75±0.16[a,b]	0.55±0.19[a,b]	1.9±0.37[a,b]	1.63±0.13[a,b]
Ghrelin and Zingerone pretreated	0.98±0.18[a,b,c]	0.89±0.11[a,b,c]	2.3±0.19[a,b,c]	1.85±0.21[a,b,c]
F	77.7	58.1	54.87	7
P VALUE	<0.001	<0.001	<0.001	<0

[a] significant in comparison to control group (p value <0.05)
[b] significant in comparison to Ischemia reperfusion group (p value <0.05)
[c] significant in comparison to Ghrelin pretreated group (p value <0.05)

Histological Results

Light microscopic examination of the control subgroups showed no detectable differences, therefore, subgroup Ia was considered as the control group. The sections stained by H&E of the control group showed regularly arranged fundic glands formed of gastric pits, isthmus, neck, and body. Musclaris mucosa and musculosa could be seen (Fig. 1a). Apical part of fundic glands lined with normal surface columnar cells with basal oval nuclei, mucous neck cells with basal flattened nuclei and parietal cells with central rounded nuclei and eosinophilic cytoplasm (Fig. 1b). Chief cells appeared with rounded nuclei and basophilic cytoplasm. Parietal cells were also noticed (Fig. 1c).

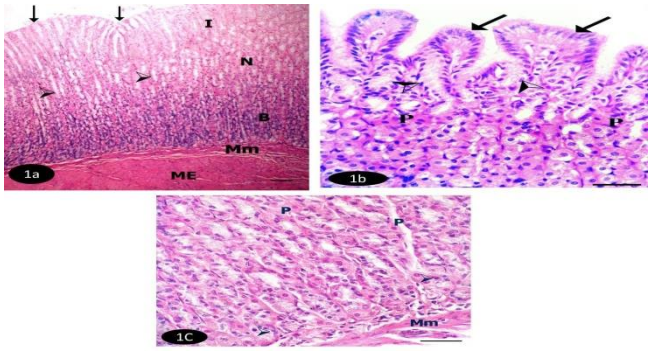

Figure 1. (H&E (a) x100 scale bar 50 µm. (b&c) x400 scale bar 30 µm), H and E stained section in the stomach fundus of adult albino rats from the control group Showing: **(1a)** regularly arranged fundic glands (arrow heads) formed of gastric pits (arrows), isthmus (I), neck (N), and body (B). Musclaris mucosa (Mm) and musculosa (ME) can be seen. **(1b)** The apical part of fundic glands lined with normal surface columnar cells with basal oval nuclei (arrows), mucous neck cells with basal flattened nuclei (head arrows) and parietal cells with central rounded nuclei and eosinophilic cytoplasm (P). **(1c**) Chief cells (head arrows) with rounded nuclei and basophilic cytoplasm, parietal cells (P), and musclaris mucosa (Mm).

The sections stained by H&E of the gastric ischemic-reperfusion group showed extensive loss of epithelial cells of mucosa and exfoliated dead cells appeared in the lumen (Fig. 2a).

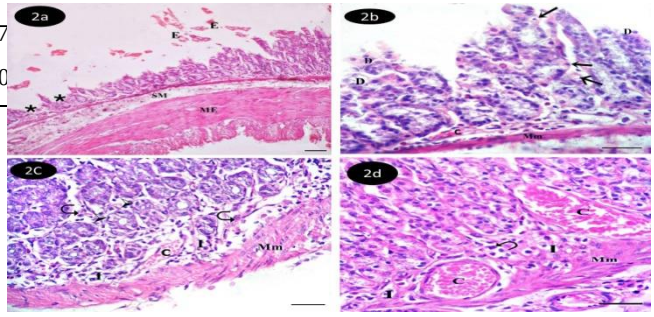

Figure 2. (H&E (a). x100 scale bar 50 µm. (b,c&d). x 400 scale bar 30 µm), H and E stained section from fundic mucosa of ischemic reperfusion group showing **(2a)** Extensive loss of epithelial cells of mucosa (asterisk). Exfoliated dead cells appear in the lumen (E). **(2b)** Severe distorted fundic glands (D). Parietal cells appear with vacuolated cytoplasm and pyknotic nuclei (arrows). Congested blood vessel (C) is also seen. **(2c**) & (2d) Vacuolated chief cells (head arrows) with pyknotic nuclei. Congested blood vessels (C) with extravasation of blood (curved arrows) and cellular infiltration (I) are also seen.

Notice, submucosa (SM), musclaris mucosa (Mm) and musculosa (ME).

Severe distorted fundic glands was noticed. Parietal cells appeared with vacuolated cytoplasm and pyknotic nuclei. Congested blood vessels were also seen (Fig. 2b). Chief cells appeared with vacuolated cytoplasm and pyknotic nuclei. Congested blood vessels with extravasation of blood and cellular infiltration were also observed (Fig. 2c&2d). However, Ghrelin pretreated group showed apparently normal mucosa consisting of packed glands. Some widening of gastric pits was also seen (Fig. 3a).

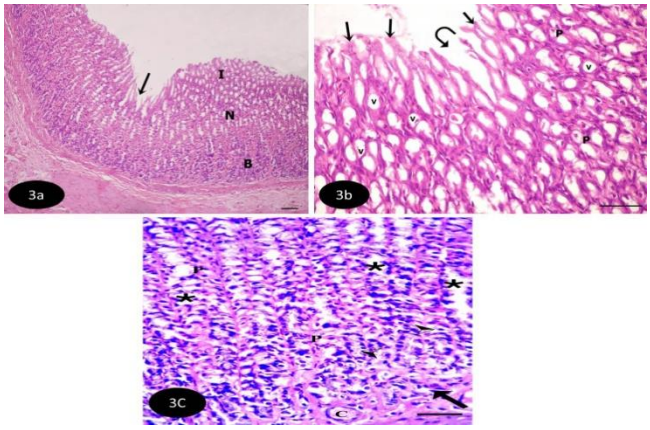

Figure 3. H&E (a). x100 scale bar 50 µm. (b&c). x400 scale bar 30 µm, H and E stained section from fundic mucosa of ghrelin pre-treated group showing **(3a)** apparently normal mucosa consisting of packed glands formed of isthmus (I), neck (N) and base (B). Some widening of gastric pits (arrow) is also seen. **(3b)** Some restoration of the surface epithelium (arrows) is seen. Most of the parietal cells (P) have vacuolated cytoplasm and pyknotic nuclei. Wide gastric pits (curved arrow) and marked vacuolations (V) of the gastric gland cells are also seen. **(3c)** Disorganization of the fundic glands (asterisk) is noticed. Numerous chief cells (head arrows) and parietal cells (P) have vacuolated cytoplasm. Congested blood capillary (c) and cellular infiltrations (arrow) are seen in lamina propria.

Some restoration of the surface epithelium was noticed. Most of the parietal cells had vacuolated cytoplasm and pyknotic nuclei. Wide gastric pits and marked vacuolations of the gastric gland cells were also seen (Fig. 3b). Some of fundic glands were disorganized with vacuolated chief cells and parietal cells. Lamina propria had Congested blood capillaries and cellular infiltrations (Fig. 3c); while ghrelin and Zingerone pretreated group showed a structure nearly similar to that of the control group with regularly arranged fundic glands and gastric pits (Fig. 4a). Surface mucous cells had oval basophilic nuclei. Mucous neck cells with flattened nuclei and parietal cells with rounded nuclei and acidophilic cytoplasm were seen. Exfoliated cells appeared in the lumen (Fig. 4b). Many chief cells and parietal cells appeared intact. (Fig. 4c).

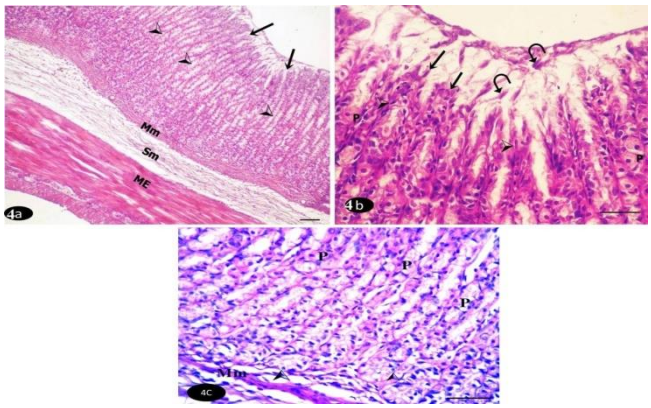

Figure 4. H&E (a). X100 scale bar 50 µm. (b&c) .x400 scale bar 30 µm, H and E stained section from fundic mucosa of zingerone and ghrelin pre-treated group showing **(4a)** structure nearly similar to that of the control group. Regularly arranged fundic glands (head arrows) and gastric pits (arrows) are observed. Musclaris mucosa (Mm), submucosa (Sm) and musclaris externa (ME) are also seen. **(4b)** Surface mucous cells (arrows) have oval basophilic nuclei. Mucous neck cells with flattened nuclei (head arrows) and parietal cells with rounded nuclei and acidophilic cytoplasm (P) are seen. Exfoliated cells (curved arrows) appear in the lumen. **(4c)** Great preservation of the fundic glands is seen. Many chief cells (head arrows) and parietal cells (P) are intact. Notice, musclaris mucosa

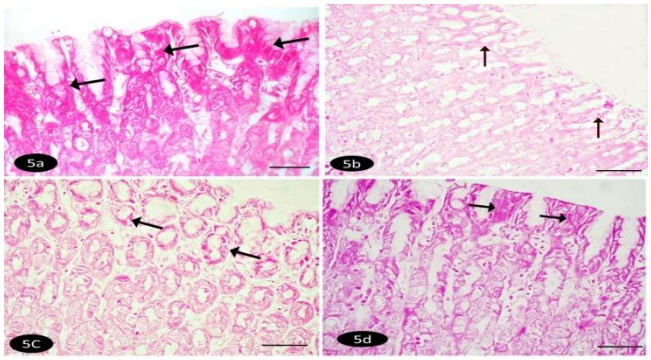

Figure 5. (PAS, X400 scale bar 30 µm), PAS stained section in the stomach fundic region of adult albino rats showing **(5a)** strong positive reaction to mucous cells lining the fundic pits (arrows) in the control group. **(5b)** The PAS reaction is weak (arrows) in ischemic reperfusion group (5C) Theghrelin pre-treated group appears with mild positive PAS reaction (arrows). (5d) The zingerone and ghrelin pre-treated group has moderate PAS reaction (arrows).

The sections stained by PAS stained showed a strong positive reaction to mucous cells lining the fundic pits in the control group (Fig. 5a). The PAS reaction was weak in the ischemic-reperfusion group (Fig. 5b). The reaction was mild in Ghrelin pretreated group (Fig. 5c) In Ghrelin and Zingerone pretreated group PAS reaction was moderate (Fig. 5d).

Immunohistochemical stains

Examination of immunohistochemically stained sections for Ghrelin-positive neuroendocrine cells showed many many positive cytoplasmic brownish reactions which located at the bottom of the gastric glands in the control group (Fig. 6a). In the ischemic-reperfusion group, few cells with Ghrelin immunoreaction was observed (Fig. 6b). In Ghrelin pretreated group, there was a mild reaction in some gastric glands (Fig. 6c). The ghrelin and zingerone pretreated group showed moderate reaction in multiple glands (Fig. 6d).

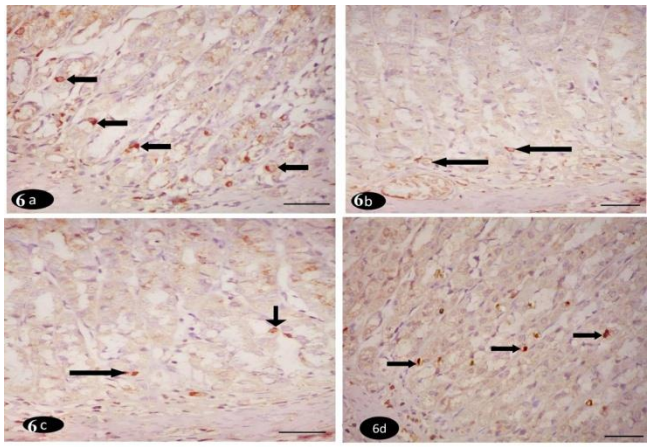

Figure 6. Anti-ghrelin immunoreaction, X400 scale bar 30 µm),A photomicrograph of the stomach fundic region of adult albino rats showing (6a) many positive cytoplasmic brownish reaction for ghrelin-secreting cells which located at the bottom of the gastric glands (arrows) in the control group. (6b) few cells (arrows) in ischemic reperfusion group. (6c) Mild in some gastric glands (arrows) in ghrelin pre-treated group . (6d) Moderate in multiple glands (arrows) in zingerone and ghrelin pre-treated groups.

The immunohistochemically stained section for TNF-α immunoreaction which appeared as cytoplasmic brownish reaction. (index for inflammation) showed a weak reaction in the control group (Fig. 7a). In ischemic-reperfusion group, highly positive immunoreaction was observed (Fig. 7b). In Ghrelin pretreated group, moderate immunoreaction was seen (Fig. 7c). In Ghrelin and Zingerone pretreated group, weak positive TNF-α immunoreaction could be seen (Fig.7d).

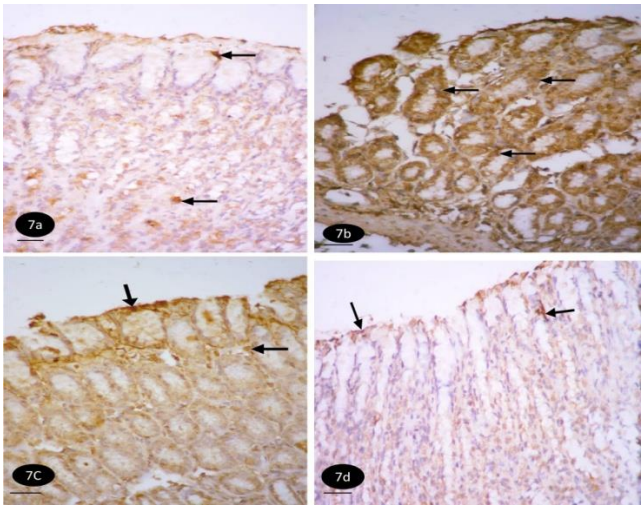

Figure 7. (TNF-α immunoreaction, X400 scale bar 30 µm) A photomicrograph of the stomach fundic region of adult albino rats showing **(7a)** weak positive TNF-α immunoreaction (arrows) in the control group, **which appears as cytoplasmic brownish reaction. (7b)** highly positive TNF-α immunoreaction (arrows) in ischemic reperfusion group**.(7c)** moderate in some gastric glands (arrows) in ghrelin pre-treated group. (7d) weak positive TNF-α immunoreaction (arrows) in ghrelin concomitant with zingerone pretreated group.

Morphometric and statistical analysis

Morphometric analysis of the mean area percentage of the PAS-positive reaction revealed a highly significant decrease in the ischemic reperfusion group compared to the control group, whereas Ghrelin pretreated group showed a significant decrease compared to the control. On the other hand, Ghrelin and Zingerone pretreated group expressed a non-significant difference from the control (Table 3).

Morphometric analysis of the mean area percentage of Ghrelin positive cells immunoreactivity showed a highly significant decrease in the ischemic-reperfusion group compared to the control one. Ghrelin pretreated group showed a significant decrease compared to the control. While Ghrelin and Zingerone pretreated group expressed a non-significant difference from the control (Table 3).

Table 3. Summarizes Mean area % of positive PAS reaction in all groups by one-way ANOVA test.

Parameter	Control Group	ischemic-reperfusion group	Ghrelin pretreated group	Ghrelin and Zingerone pretreated group	P
the mean valu of the area % positive PAS reaction	29.45±1.73	12.22±0.64**	24.94±4.78*	27.87±4.42	<0.001**

Values are expressed as mean ± standard deviation (SD). **: Highly significant (P<0.001).

Table 4. Summarizes Mean area % of ghrelin-positive cells immunoreactivity in all groups by one-way ANOVA test

Parameter	Control Group	ischemic-reperfusion group	Ghrelin pretreated groups	Ghrelin and Zingerone pretreated group	P
the mean values o the area % of ghrelin positive cells immunoreactivity	25.13 ±6.21	13.20 ±5.10**	17.13 ± 7.75	22.87±4.32	<0.001**

Values are expressed as mean ± standard deviation (SD). **: Highly significant (P<0.001).

Table 5. Summarizes the mean values of the area % of TNF α immunoreactivity ±SD in all groups by one-way ANOVA test.

Parameter	Control Group	Ischemic reperfusion Group	Ghrelin pretreated group	Ghrelin and Zingerone pretreat group	P
the mean values o the area % of TNF immunoreactivity	2.01 ±0.71	19.98±2.2	8.2±1.3	6.97±1.2	<0.001**

Values are expressed as mean ± standard deviation (SD). **: Highly significant (P<0.001). significant (P<0.05).

Morphometric analysis of the mean area percentage of TNF-α immunostaining was highly significantly increased in the ischemic-reperfusion group compared to the control group (P< 0.01). On the other hand, it was a highly significant decrease in Ghrelin and Zingerone pretreated group and the Ghrelin pretreated groups compared to the ischemia-reperfusion group (P<0.01). While there was a significant decrease in the mean area percentage in Ghrelin and Zingerone pretreated group compared to Ghrelin pretreated group (P<0.05).

Discussion

Gastrointestinal ischemia-reperfusion results from blood deprivation and inhibits the aerobic metabolism so, forcing the cells to perform anaerobic metabolism that causes promotion damage to the tissue (20). The damage occurs with the arrival of oxygen, cytokines, reactive oxygen species (ROS), neutrophils and the alteration of capillary permeability. Inactivation of these molecules before tissue reperfusion could minimize gastrointestinal damage (21, 22).

Gastric ulcer induced by stress is a serious injury occurred after sepsis, major surgery, burns, or trauma to the central nervous system (23). In this work, the gastric ulcer was experimentally made using the ischemic and reperfusion operation to the stomach; similar study was previously carried out by Brasilerio et al (24). The sections stained by H&E of the ischemic-reperfusion group showed the affection of the mucosa of the stomach fundus in the form of vacuolation, pyknosis of fundic gland cells, ulceration and deep erosions with the shedding of the superficial epithelial cells. These results were proved also in PAS-stained sections of this group, which showed a reduction of mucus secreted cells. Similar results were detected by Odukanmi et al. (25). These microscopical changes were explained by Seino et al. (26). They mentioned that many agents like cytokines, reactive oxygen species (ROS) and bioactive amines have been involved in stress ulcer formation.

Gastric mucosal hyperemia, blood extravasation, and cell infiltration were also found in the study. These results are in agreement with those detected by Rocha et al. (27).

Tóth and his colleagues stated that long-term ischemia and reperfusion of the jejunum can cause damage to the integrity of the intestinal barrier and subsequent massive translocation of endotoxin. Other researchers proved that these phenomena cause an inflammatory reaction that includes complement

activation, endothelial activation, neutrophil sequestration and the release of inflammatory mediators into the blood (28, 29).

In the examination of the immunohistochemically stained section of the ischemic-reperfusion group, there was increased TNF-α expression. This result was statistically proved by the highly statistical difference in this group compared to the control one. More or less analogous results were detected in ethanol-induced ulcers. The increase in the levels of different cytokines as IL-6, IL-8 and TNF-α and chemokines are directly chemotactic to leukocytes that are key regulators of inflammatory processes. Moreover, the disorders of gastric mucosal barriers may lead to bacterial infection. These microorganisms have a chemotactic factor for mononuclear inflammatory cells leading to gastritis and mucosal erosion (30, 31). Moreover, the cytokines had a vulnerable role in the stimulation of fibroblasts and transforming them into myofibroblasts that initiate fibrosis (32).

Immunohistochemical outcomes of our study revealed a statistically significant decrease in the average number of ghrelin-positive cells at the base of the gastric glands in the ischemic-reperfusion group compared to the control group. This reduction occurs as part of mucosal damage due to ischemic-reperfusion.

Sibilia et al. (33) demonstrated a probable role of Ghrelin in protective mechanisms of gastric mucosa, resulting in significant gastroprotection against ethanol-induced lesions. Furthermore, numerous research conducted by Brzozowski et al (34) proposed that Ghrelin had substantial protective benefits against acute stomach mucosal damage caused by stress and that these effects were mediated through vagal neurons, sensory nerves, and the NO system. The current study found that Ghrelin inhibits acute stomach damage caused by I/R and also speeds up the curing of these lesions. The mechanism through which Ghrelin can promote repair of I/R-induced injuries is unknown. Other research has shown that growth factors that promote angiogenesis have a great role in the healing mechanism (35). According to Baatar et al. (36), Ghrelin-accelerated curing of acute I/R - induced lesions was associated with increased expression of HIF-1, which is ascribed to enhanced production and activation of VEGF. In our investigation, the association of Ghrelin and I/R definitely increased the production of HIF-1 and VEGF. A considerable reduction in the stomach mucosal lesions induced by I/R was recorded, since Ghrelin increased the production of HIF-1 and VEGF. However, Konturek et al. (37) show that Ghrelin's early recovery from I/R damage is perhaps related to increased production of HIF-1, while delayed recovery may entail both HIF-1 and VEGF overexpression. The early reduction in VEGF expression, despite raised HIF-1 expression twenty-four hours after I/R damage, might be attributable to raised production of metabolites of free oxygen and lipid peroxidation, both of which can limit VEGF excitation. However, repair of I/R-induced gastric injuries requires the activation of antioxidant enzymes, which inhibits the formation of free oxygen species and leads to an increase in mRNA expression of VEGF (38, 39).

When ROS outnumber antioxidant enzymes, oxidative stress occurs. Cells fight themselves against oxidative stress by producing enzymes such as SOD, glutathione peroxidase and catalase. Nrf2 is the protective mechanism involved in oxidative and chemical stress; when exposed to electrophilic or pro-oxidative stimuli, Nrf2 is emitted into the nucleus. Many antioxidant genes are activated by Nrf2, which helps to maintain cellular homeostasis (40, 41). It is widely documented that Nrf2 signalling primarily regulates the transcription of those antioxidant genes via NO. that is produced by NOS enzymes through the oxidation of Larginine. eNOS is the primary source of circulating NO (42).

We discovered that Zingerone reduces oxidative stress after ischemia stress by signalling through the eNOS/NO/Nrf2 pathway. In this investigation, light microscopic examination of the stomach fundus group treatment with Ghrelin concurrently with Zingerone verified this. They had a structure that was virtually identical to the control group, with consistently placed fundic glands and stomach pits. PAS-stained sections from the same group reacted moderately as compared to the week ones from the ischemia/reperfusion group. The control group's immunohistochemical stained sections for TNF- immunoreaction (an indicator of inflammation) revealed a modest reaction. A substantially positive immunoreaction appeared in the ischemia-reperfusion group. Moderate

immunoreactivity was noticed in the Ghrelin-treated group. A mild positive TNF- immunoreaction was observed in Ghrelin plus Zingerone group.

It is generally understood that NO plays a role in Nrf2 activation. NO stimulates translocation of Nrf2 from the cytoplasm into the nucleus and increases antioxidant response element (ARE), enhancing various antioxidant enzymes transcription such as glutathione S-transferase (GST), glutathione peroxidase 1 (GPx1), NAD(P)H quinone oxidoreductase 1 (NQO1), SOD, and HO1. Activation of the eNOSNrf2Tfam pathway may result in mitochondrial biogenesis (43, 44). Zingerone stimulates eNOS, boosting NO generation and inhibiting the transcription of Nrf2associated antioxidant genes. However, several studies have found that Nrf2-mediated gene expression and NOS activity are regulated in ways that contradict our findings. According to Heiss et al., research showed that activated Nrf2 is due to eNOS coupling by establishing stoichiometric equilibrium between BH4 and eNOS and that it increased NO bioavailability but transiently lowered eNOS levels in primary endothelial cells (45, 46). Erkens et al. discovered that, despite lower antioxidant reserve capacity, Nrf2 deficiency upregulated expression of eNOS and had protective benefits against I/R damage. The result might be related to differences in regulation of Nrf2 and eNOS or NO in various cells, tissues, illnesses, models and ages of mice (47).

One of the primary regulators of hypoxia/ischemia is hypoxia-inducible factor-1 alpha (HIF-1a). HIF-1a activates multiple gene transcription, including angiogenic vascular endothelial growth factor (VEGF) which is an important growth factor for angiogenesis (48, 49). VEGF promotes vessel endothelial cell proliferation, inhibits endothelial cell apoptosis, and stimulates blood vessel formation (50).

Conclusion

Based upon results of the current study, it could be concluded that after acute gastric ischemia for 30 minutes, then reperfusion for 24 hours ischemia reperfusion injury, pretreatment with Ghrelin in concomitant with Zingerone is superior to pretreatment with Ghrelin alone. Ghrelin in concomitant with Zingerone have great protective role on gastric mucosal injury. This was through its potent anti-inflammatory and antioxidant effects beside which was evidenced through gene expression changes, histologically and immunohistochemically. . **So, we recommend** increasing the intake of ginger in our food and encouraging further research studies on this vital antioxidant.

Conflict of interest

There is no potential conflict of interest among the authors.

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Ethical approval

All animal experiments were carried out in accordance with relevant guidelines and regulations of the Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC committee).

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