The postulated mechanism of the protective effect of ginger on the aspirin induced gastric ulcer: Histological and immunohistochemical studies

Mahmoud Salah Khalil

Department of Histology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
and Medical College, King Saud University, Riyadh, KSA

Summary. There are many available drugs for treating gastric ulcer, but they have various side effects. Ginger is a folk, herbal medicine, which is used for treatment of various diseases including gastric ulcer. This study investigates the possible mechanism of the protective effect of ginger on aspirin induced gastric ulcer. Forty adult male albino rats were randomized into four groups (10 animals per each group) and orally received the followings once daily for 5 days: Group I: 3 ml of 1% carboxymethyl cellulose; Group II: ginger powder (200 mg/kg body weight) suspended in 3 mL of 1% carboxymethylcellulose; Group III: aspirin (400 mg/kg body weight) suspended in 3 ml of 1% carboxymethylcellulose in water. Group IV: ginger and 30 minutes later, received aspirin suspended in 1% carboxymethylcellulose, in similar doses as received in groups II and III. On day 6, rats were sacrificed. The animals were anesthetized and the stomach was removed for the macroscopic, histological (Haematoxylin and Eosin and Periodic Acid Shiff) and immunohistochemical investigations (Bax, inducible nitric oxide synthase and heat shock protein 70).

Aspirin induced a significant increase of the macroscopic ulcer score, shed and disrupted epithelium, mucosal hemorrhage, submucosal edema and leukocyte infiltration, loss of the mucus of the mucosal surface significantly increased expression of apoptosis regulator Bax, inducible nitric oxide synthase (iNOS) and heat

shock protein 70 (HSP70). Ginger ameliorated the histological changes by reducing Bax and iNOS and increasing HSP70 expressions.

Key words: Ginger, Aspirin, Gastric ulcer, Histological, Immunohistochemical

Introduction

Gastric ulcer is one of the commonest gastrointestinal disorders that penetrate the mucosa and muscularis mucosa as a result of exposure of the stomach lining mucosa to acids (Onasanwo et al., 2011). Nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, induce gastric ulcers in 10- 25% of patients (Tibble et al., 2001). Aspirin is a potent NSAID that is widely used for the treatment of rheumatoid arthritis and prevention of cardiovascular thrombotic diseases. Other NSAIDs, such as indomethacin, induce ulcers through directly damage to the gastric mucosa (Kauffman, 1989), decreasing prostaglandin synthesis (Wang et al., 1989; Pawlik et al., 2002; Laine et al., 2008), inhibiting ulcer contraction (Hirose et al., 1991) and decreasing mucosal blood flow of the gastric ulcer margin (Hirose et al., 1991). The increase in gastric pro-inflammatory cytokines, including interleukin-1 and tumor necrosis factor (TNF) plays a role in the pathogenesis of gastric mucosal damage (Wang et al., 2007; Wallace, 2008) through the recruitment of white blood cells which is accompanied by increased TNF-production (Naito et al., 2001; Jainu and Devi, 2006) augmenting the generation of the neutrophil-derived

superoxide (Kwiecien et al., 2002) and stimulating IL-1 production, leading to more recruitment of neutrophil (Kokura et al., 2000; Odashima et al., 2006). The reactive oxygen species (ROS) is one of the important factors in inducing mucosal damage via oxidative damage of the cellular membrane and intracellular molecules (Kanter et al., 2005).

Increases in nitric oxide synthase (NOS) activities, especially inducible nitric oxide synthase (iNOS) are also involved in the pathogenesis of gastric mucosal damage (Muscara and Wallace, 1999; Wallace and Miller, 2000). Heat shock protein 70 (HSP70) however, plays an important role in gastric mucosal protection (Shen et al., 2001). HSP70 is known to improve cellular recovery by refolding partially damaged functional proteins and increasing delivery of precursor proteins to important organelles such as mitochondria and endoplasmic reticulum, which may result in complete, efficient mucosal defense mechanisms and achieve ulcer healing (Choi et al., 2009).

Although there are many available drugs used for the treatment of gastric ulcer, they produce various side effects such as arrhythmias, impotence and hematopoietic changes (Rang et al., 2003). Ginger, a folk medicine, is generally considered a safe herbal medicine (Weidner and Sigwart, 2001). Ginger has been reported to treat a variety of diseases such as arthritis, rheumatism, sprains, muscular aches, pains, sore throats, cramps, constipation, indigestion, vomiting, hypertension, dementia, fever, infectious diseases and helminthiasis (Dedov et al., 2002; Jiang et al., 2005; Ali et al., 2008). Ginger has also been demonstrated to have a glucose- and lipid-lowering effect (Shukla and Singh, 2007; Nicoll and Henein, 2009) and protects against drug induced gastric mucosal injury (Wang et al., 2011).

Ginger powder contains 6-gingerol, 6-shogaol, 8-gingerol and 10-gingerol (Schwertner et al., 2006). The major ginger constituents that include 6-gingerol and 6-shogaol have been reported to have anti-oxidant, antitumor and anti-inflammatory effects (Surh, 2002; Kim et al., 2005; Young et al., 2005). 6-Shogaol, a dehydration product of 6-gingerol, is found in ginger powder but is not found in fresh ginger. 6-Shogaol appears to be formed from 6-gingerol during the heating processing and long term storage (Nikam et al., 2013).

The mechanism underlying the protective effects of ginger against gastric damage is unclear. On a histological and immunohistochemical basis, this study investigates the mechanism of antiulcer effect of ginger on aspirin-induced gastric ulcer in model rats.

Materials and methods

A total of 40 adult Wistar male albino rats (200-250 g) were maintained with water and food ad libitum at constant humidity and temperature. The animals were randomized into 4 groups (10 animals per each group) and orally (through oral gavage) received the following once daily for 5 days.

Group I (control 1): 3 ml of 1 % carboxymethyl cellulose in water.

Group II (control 2): ginger powder (200 mg/kg body weight), suspended in 3 ml of 1% carboxymethyl-cellulose in water (Wang et al., 2011; Auti and Kulkarni, 2013).

Group III (aspirin treated group): after 24 h fasting, rats received aspirin. (400 mg/kg body weight) suspended in 3 ml of 1% carboxymethylcellulose in water (Raghavendran et al., 2011; Wang et al., 2011).

Group IV (aspirin and ginger treated group): after 24 hr fasting, rats orally received ginger similar to group II. 30 minutes later, the rats received aspirin similar to group III.

On day 6, rats of all groups were sacrificed.

The animals were anesthetized by ether inhalation. The stomach was then cut open along the greater curvature. The stomach was removed, washed with warm saline and underwent the macroscopic, histological and immunohistochemical investigations.

The animal manipulations were performed in the Laboratory Animal Center of College of Medicine, King Saud University (Riyadh, KSA) in accordance with the institutional and national guide for the care and use of laboratory animals. The experiment was approved by The Ethical Committee of College of Medicine, King Saud University.

Macroscopic lesion scores

The macroscopic lesion injury of each ulcer was investigated by stereomicroscope (LEICA, L2, Germany) and was scored by two independent observers to determine the lesion score scale which ranged from 0 to 4: (0) no macroscopic changes, (1) mucosal erythema only, (2) mild mucosal edema, slight bleeding or small erosions, (3) moderate edema, bleeding ulcers or erosions, and (4) severe ulceration, erosions, edema and tissue necrosis (Millar et al., 1996).

Histological and immunohistochemical studies

The stomach of each rat was dissected and fixed in 10% neutral buffered formalin solution (Sigma Chemical Co., St. Louis, MO) for 24 hours. The stomach specimens were processed to prepare 4 μm-thick paraffin sections. The sections were stained for histological and immunohistochemical studies.

Histological studies

Haematoxylin & Eosin (H&E) (Drury and Wallington, 1983) and periodic acid Schiff (PAS) (Cook, 1974) were used for histological and mucin histochemical reaction investigations respectively.

Immunohistochemical studies

Paraffin sections were processed for Bax (B-9), inducible nitric oxide (iNOS, sc-7271), and heat shock protein (HSP70, sc-24) antibodies which were purchased

from Santa Cruz, USA. After deparaffinization in xylene, immunohistochemistry was performed using a 3-step indirect process based on the labeled avidin biotin peroxidase complex (ABC) method. Sections were rehydrated in descending grades of alcohol. Following blocking of endogenous peroxidase activity with 3% H_2O_2 in methanol and non-specific binding sites with a protein blocker, the sections were incubated for 32 minutes with a 1:100 dilution of the primary antibodies (Rabbit anti-rats Bax, iNOS and HSP70 polyclonal antibody, Santa Cruz, USA). Then, biotinylated secondary antibody was added at a concentration of 2% for 30 minutes (37°C) followed by the addition of the avidin-biotin-peroxidase complex (ABC). Visualization of the reaction was performed using 3, 3-diamino-benzidine (DAB) as the chromogen which produces a dark brown precipitate that was readily detected by light microscopy. The sections were then counterstained with Mayer's hematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX (the Bax, iNOS and HSP70 cytoplasmic reactions were stained brown). The negative control included sections which were incubated in the absence of the primary antibody. The sections were independently examined by the author and a pathologist.

Quantitative analysis

Quantitative measurements were carried out using an image analyzer (Super eye-Heidi soft, Histology Department, Faculty of Medicine, King Saud University, Saudi Arabia). Fifteen high power fields (Objective, X, 400) were captured for each animal. The mean optical density (OD) of Bax, iNOS and HSP70 cytoplasmic brown color reactions stomach mucosa was measured. The image analyzer was calibrated for color measurement before using.

Statistical analysis

The data was analyzed using SPSS statistical software version 19. The values were expressed as Mean±SEM. The means of color area percent and OD of the immune-positive immunohistochemical reactions among the studied groups were compared using ANOVA-test. The significance level was set at a p value

≤ 0.05.

Results

The macroscopic ulcer score was significantly higher (p for ANOVA =0.000) in group III (aspirin treated group) compared to the other groups (Table 1).

Histological and immunohistochemical studies

The H&E histological staining of the control groups (I and II) showed the normal structure of the stomach (Fig. 1A,B) with PAS positive reaction on the surface of the mucous which extended down to the gastric pits (Fig. 1C). The Bax and HSP70 immuno-histochemical reactions were not expressed (Fig. 1D,E) but the iNOS was minimally expressed in the gastric mucosa (Fig. 1F).

In aspirin treated group (group III), aspirin induced shed and disrupted epithelium, mucosal hemorrhage, submucosal edema and leucocyte infiltration (Fig. 2A,B). The PAS reaction showed loss of mucus in the majority of the investigated gastric mucosal surface (Fig. 2C). Bax and iNOS were markedly expressed (Fig. 2D,E) and HSP70 was moderately expressed in the stomach mucosal cells (Fig. 2F).

In aspirin and ginger treated group (group IV), the microscopic pictures showed less histological changes in terms of shed and disrupted epithelium in addition to the submucosal leucocyte infiltration (Fig. 3A,B). The PAS mucosal reaction was restored (Fig. 3C). Bax and iNOS were moderately expressed (Fig. 3D,E) and HSP70 was markedly expressed in the stomach mucosa (Fig. 3F).

The quantitative analysis of the Bax and iNOS immunohistochemical reactions was significantly

Table 1. The measurement of ulcer index scores.

| Groups	Ulcer index scores (mean±SEM)	P value for ANOVA
Group I (Control 1)	0±0	0.000
Group II (Control 2)	0±0	
Group III (Aspirin treated group)	2.83±0.4*	
Group IV (Aspirin and ginger treated group)	1.17±0.4	

*, Significant p value compared to each group.

Table 2. Optical density measurement (Mean of the AU± SEM) of Bax, HSP70 and iNOS protein in the gastric mucosa of different groups.

	Group I	Group II	Group III	Group IV	p value for ANOVA test
Bax	0.031±0.006	0.029±0.007	0.21±0.02*	0.12±0.02	0.000001
HSP70	0.04±0.0008	0.05±0.0009	0.11±0.02	0.21±0.03*	0.000005
iNOS	0.005±0.0007	0.004±0.0006	0.258±0.03*	0.044±0.03	0.000001

AU, arbitrary unit; OD, optical density; *, significant p value compared to each group.
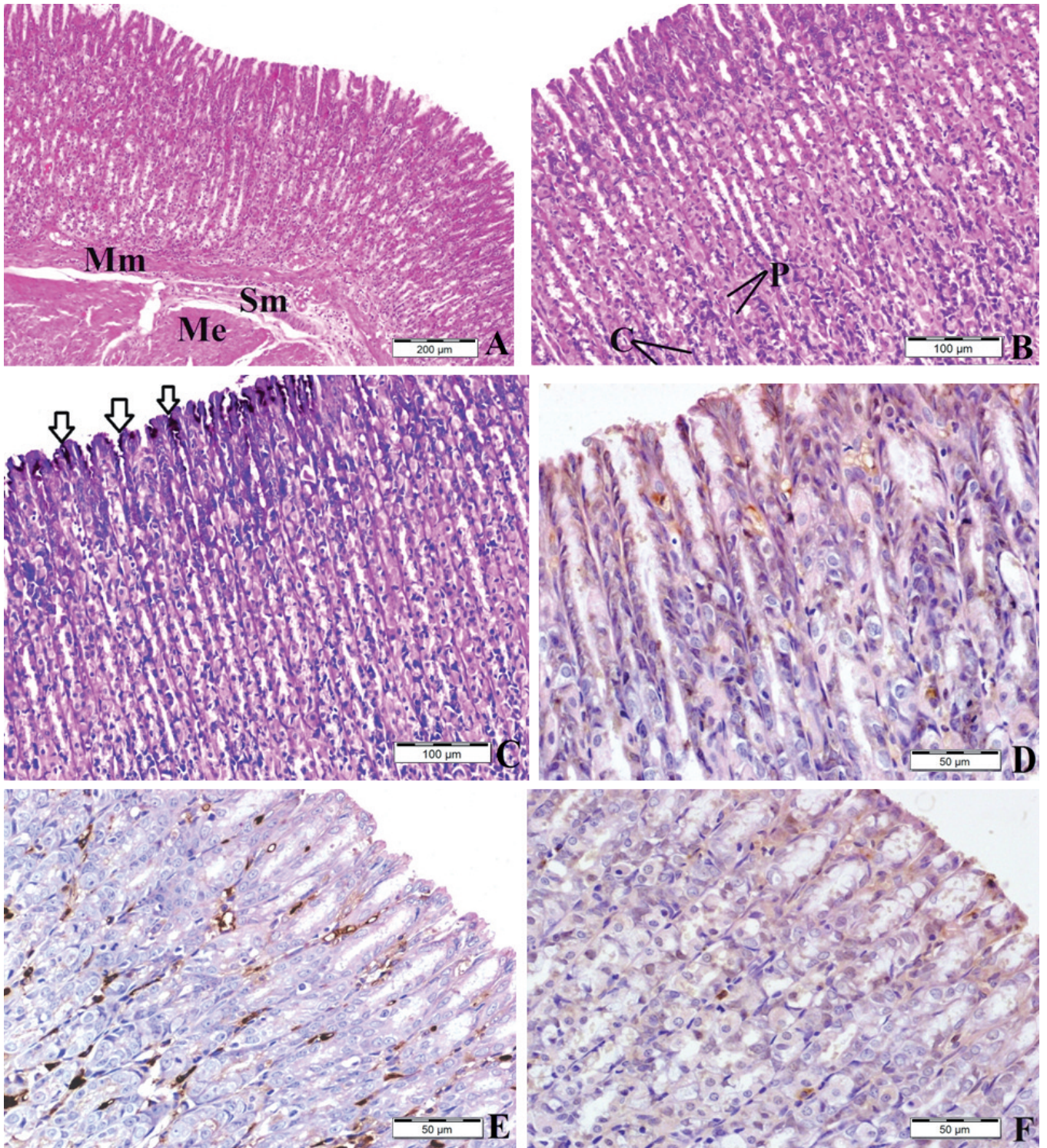
Fig. 1. Control stomach. **A.** Muscularis mucosa (Mm); Submucosal (Sm); Muscularis externa (Me) (H&E). **B**. Parietal cells (P); Chief cells **(C)** (H&E). **C.** Positive PAS stain reactions (Arrows) (PAS). **D.** Bax Immunostaining (DAB&H). **E.** Heat shock protein immunostaining (DAB&H). **F.** iNOS immunostaining (DAB&H).
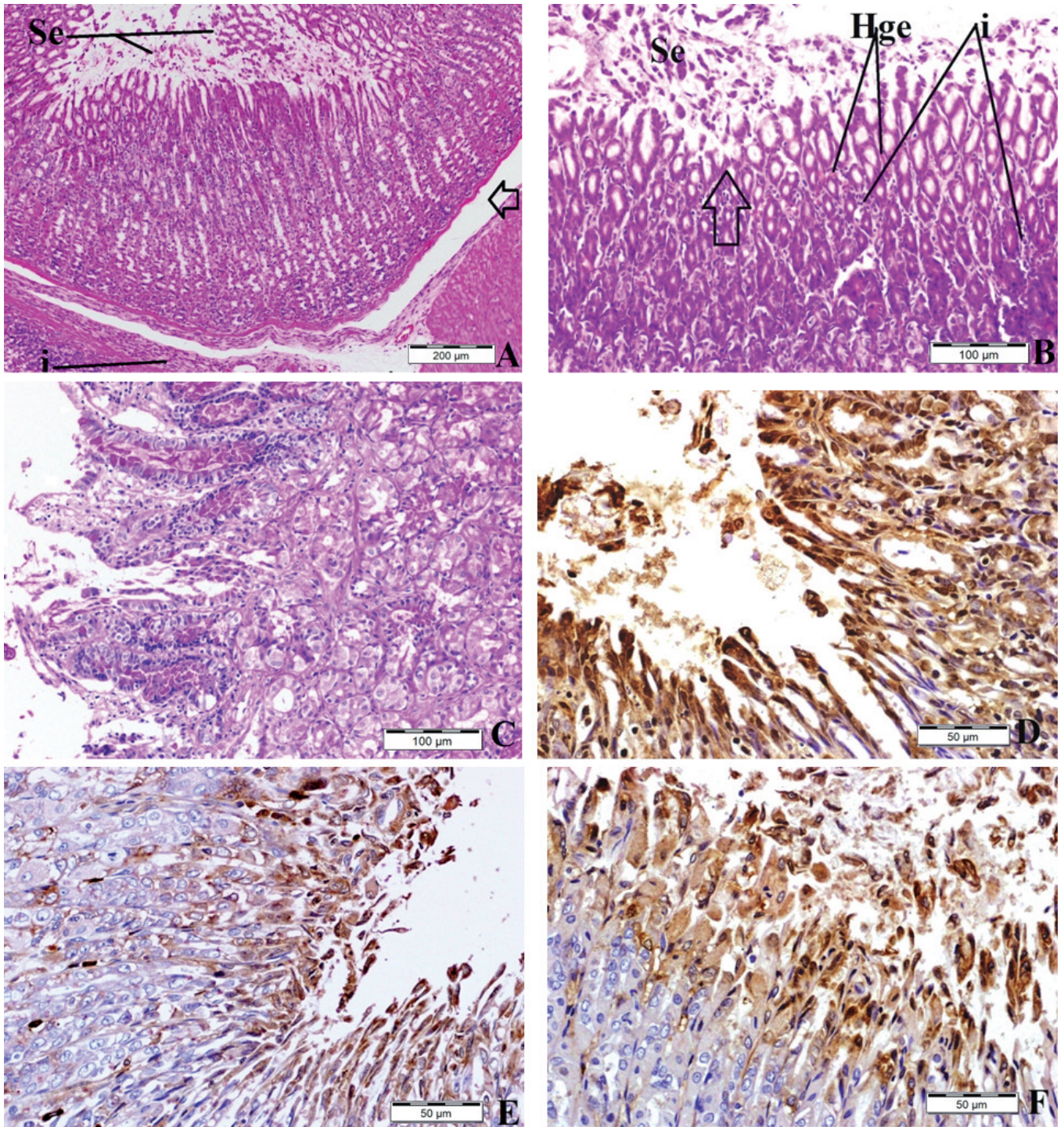
increased in group III compared to the other groups.

HSP70 however, was significantly higher in group IV compared to groups I, II and III (Table 2).

Discussion

The anti-ulcerative effects of ginger have previously been investigated in experimental gastric ulcer models

Fig. 2. Aspirin treated stomach. **A.** Shed epithelium (Se); Submucosal edema (Arrow); Submucosal leucocytic infiltration (i) (H&E). **B.** Shed epithelium (Se); Hemorrhage (Hge); Leucocytic infiltration (i); Disrupted mucosa (Arrow) (H&E). **C.** PAS stain reactions (PAS). **D.** Bax Immunostaining (DAB&H). **E.** Heat shock protein immunostaining (DAB&H). **F.** iNOS immunostaining (DAB&H).
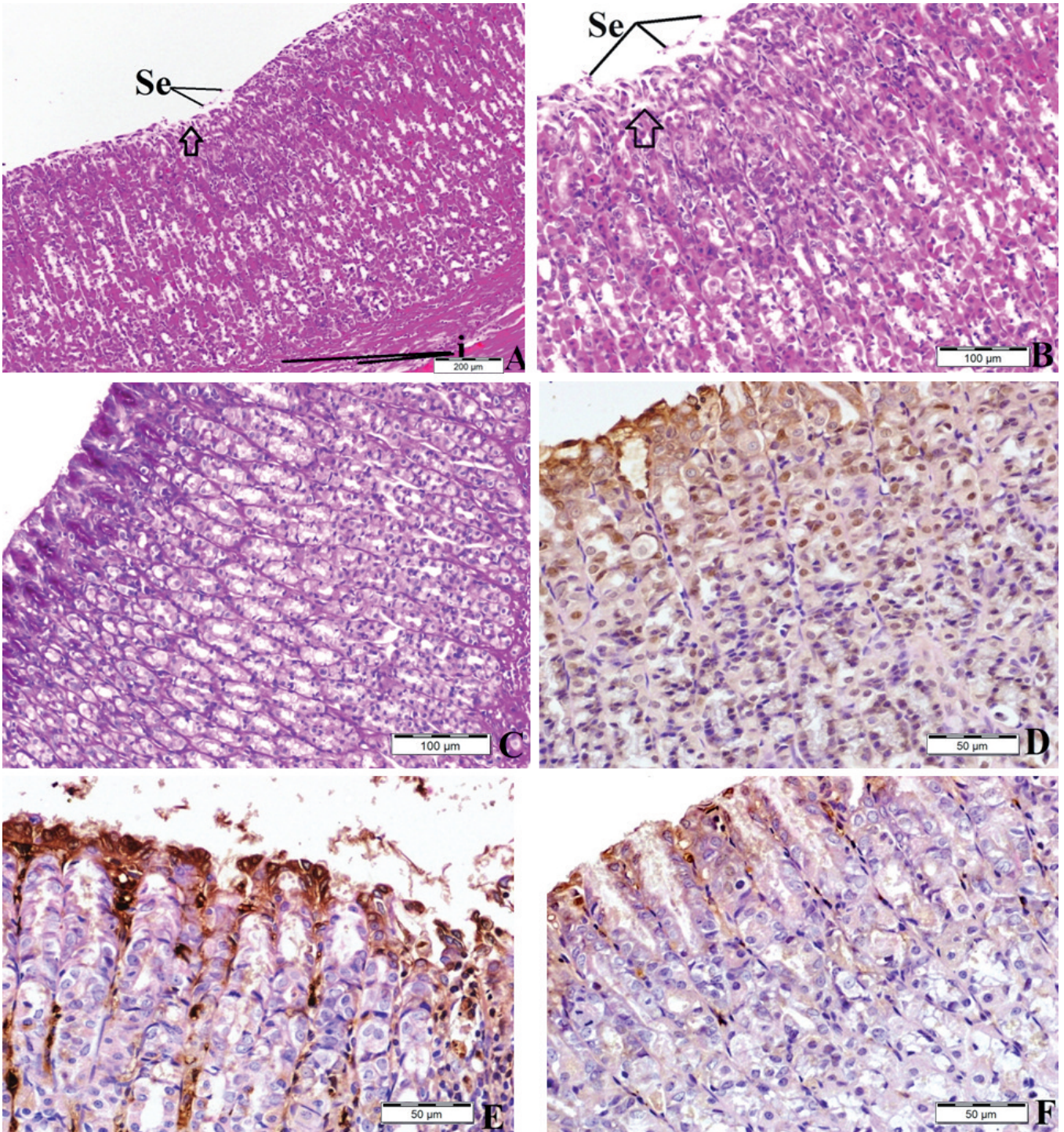
Ginger protects against aspirin induced gastric ulcer

(Yamahara et al., 1988; Khushtar et al., 2009) although the mechanism of its protective effects is unclear. In this study, the gastroprotective activity of ginger was investigated on the aspirin-induced gastric ulcer model in rats.

In the present study, macroscopic ulcer score results

Fig. 3. Aspirin and ginger treated stomach. **A.** Shed epithelium (Se); Disrupted epithelium (Arrow); Submucosal leucocytic infiltration (i) (H&E). **B.** Shed epithelium (Se); Disrupted epithelium (Arrow) (H&E). **C.** PAS stain reactions (PAS). **D.** Bax Immunostaining (DAB&H). **E.** Heat shock protein immunostaining (DAB&H). **F.** iNOS immunostaining (DAB&H).

showed that aspirin induced significant gastric mucosal changes. Microscopically, the investigations of the H&E stained sections showed that aspirin induced shed and exfoliated mucosal cells, disrupted mucosal gastric epithelium loss of mucus, mucosal hemorrhage, submucosal edema and leucocyte infiltration. These findings were in agreement with other studies which showed the aspirin effect on gastric mucosa. Aspirin significantly delayed ulcer healing and inhibited mucosal regeneration (Caselli et al., 1995), induced extravasations of erythrocytes and congestion of the blood gastric capillaries mucosa in rats (Zanardo et al., 2006).

The shed and exfoliated cells of gastric mucosa after aspirin administration might be considered a defense mechanism of the stomach against aspirin (Wallace et al., 1995).

Cellular infiltration observed in the submucosa of the aspirin treated rats had been previously demonstrated by others who attributed that to the breaking effect of aspirin on the intercellular junctions' integrity and the inhibition of the production of protective mucus layer (Sakai et al., 1997). This process directly exposes acid and proteolytic exzymes to the gastric mucosa with subsequent bacterial invasion (Sakai et al., 1997). These bacteria are chemotactic for inflammatory cellular infiltrates such as neutrophil, lymphocyte and macrophage which results in generation of reactive oxygen species (ROS). Myeloperoxidase is the neutrophil enzyme which catalyzes the oxidation of the chlorine molecule (Cl_2) by hydrogen peroxide (H_2O_2) to form hypochlorous acid (HClO) and free radicals, resulting in an acute inflammation in the gastric tissue (Halliwell and Gutteridge 2006; Fialkow et al., 2007).

In the present study the investigations of the PAS stained sections showed that a decreased intense magenta color in the surface epithelial cells of the aspirin treated groups indicated loss of the glycoprotein accumulation in the gastric mucosa in the aspirin treated group. Aspirin induced gastric injury might be due to a decreased mucous cell number leading to a disrupted natural gastric mucus barrier which protects the underlying epithelial cells from digestive proteolytic enzymes (Wallace and Tigley, 1995). Aspirin also induces a suppression of cycloxygenase enzyme, resulting in inhibition of prostaglandin synthesis. Thus, aspirin inhibits mucus and bicarbonate secretion, mucosal blood flow, epithelial cell turnover and repair, the rate of renewal of mucus barrier and alters the quality of the secreted mucus (Wallace and Tigley, 1995; Wang et al., 2007).

The immunohistochemical examination of the aspirin treated group of the present study showed that Bax and iNOS were markedly expressed and the HSP70 was moderately expressed in the gastric mucosa.

The increased iNOS expression was in agreement with another study which showed that aspirin damaging the gastric mucosa is attributed to the increased production of NO (Konturek et al., 2006). The high quantity of nitric oxide (NO) produced by iNOS damages the epithelium (Piotrowski et al., 1999; Konturek et al., 2006; Whittle, 2003; Hsu and Liu, 2004) while inhibiting aspirin-induced increases in iNOS expression in the gastric mucosa, leading to a reduction in gastric mucosal damage (Konturek et al., 2006).

The increased Bax expression was in agreement with others (AlRashdi et al., 2012; Golbabapour et al., 2013). Apoptosis is considered one of the main factors that contribute to gastric ulcer formation (Konturek et al., 1999). The increased Bax expression is a marker for apoptosis (Liu et al., 2008; Hasnan et al., 2010) which could be attributed to direct aspirin induced mitochondrial injury and subsequent cellular injury (Laine, 1996). Aspirin diffuses into the cytoplasmic gastric mucosal epithelial cells and reaches the mitochondria, which are the intracellular target organelle (Laine, 1996). Aspirin uncouples the oxidative phosphorylation to dissipate the mitochondrial transmembrane potential, leading to recruit Bax protein from the cytoplasm. Hence it releases the cytochrome c from mitochondrial intermembranous space into the cytosol resulting in generation of ROS such as superoxide and hydrogen peroxide thus causing caspase 9 and caspase 3 activation and cellular apoptosis (Brzozowski et al., 2000; Tsutsumi et al., 2002; Brand et al., 2004; Nagano et al., 2005).

The increased HSP70 expression in group III compared to control groups is in agreement with other studies that demonstrated finding of increased HSP70 proteins in ethanol induced gastric ulcer in rats (AlRashdi et al., 2012; Golbabapour et al., 2013). The HSP70 is produced in response to different cellular stresses (Oberringer et al., 1995; Tytell and Hooper, 2001).

The present study showed that ginger ameliorated the histological deleterious effect of aspirin in group IV at macroscopic and microscopic levels.

The H&E stain results showed that ginger reduced the deleterious effects induced by aspirin, including reduced submucosal edema and cellular infiltration. This result was confirmed by others who showed that ginger has an anti-inflammatory effect (Grzanna et al., 2005; Ko and Leung, 2010). The gastro-protection induced by ginger may be partially attributed to the inhibition of neutrophil infiltration and subsequent myeloperoxidase and free radical generation. The anti-inflammatory effects of ginger are attributed to inhibition of PGE2, iNOS, COX-2 and TNF- α induced by 6-gingerol and 6-shogaol (Lantz et al., 2007; Isa et al., 2008).

The PAS staining results of the present study revealed the capability of ginger to maintain gastric mucus integrity against aspirin induced depletion. Ginger participates in controlling the production and nature of the mucus in order to protect the gastric mucosa from the noxious effect of ROS formation due to aspirin administration (Khushtar et al., 2009). The increased gastric mucus secretion decreases the stomach wall friction during peristalsis, improving the buffering

of gastric acid and provides an effective barrier to back diffusion of hydrogen ion (Venables, 1986).

The immunohistochemical investigations of this study revealed a remarkable up-regulation of HSP70 and down-regulation of Bax and iNOS in the aspirin and ginger treated groups (group IV) compared to group III.

The reduced expression of iNOS was confirmed by other studies that showed that ginger powder reduced iNOS activity and inhibited aspirin induced gastric ulcers (Wang et al., 2011). The increased HSP70 expression is attributed to the fact that ginger may have a protective effect of oxidative stress due to aspirin by acting as a molecular chaperone that inhibits intracellular proteins from aggregation, thereby preserving the protein structure and allowing them to refold into their active conformation (Tytell and Hooper, 2001; Mayer and Bukau, 2005; AlRashdi et al., 2012). There is a positive correlation between HSP70 induction and mucosal protection (Yeo et al., 2008). HSP70 proteins defend cells from oxidative stress and/or heat shock. The ROS acts through inhibition of HSP70 expression and increases the expression of Bax (Golbabapour et al., 2013). HSP70 prevents the partially denatured proteins which were induced by the ROS from aggregating and allows them to refold. The remarkable expression of HSP70 noticed in this study could suggest that ginger protected the gastric tissues through its up-regulation. These proteins are responsible for the protection of the cellular homeostatic processes from environmental and physiologic injuries by preserving the structure of normal proteins and repairing or removing damaged proteins (Tytell and Hooper, 2001). Furthermore, HSP70 has its cyto-protective action by protecting mitochondria and by interfering with the stress-induced apoptotic program (Rokutan, 2000) which may explain the decrease in the expression of Bax in the present study. Overexpression of HSP70 also provides protection against other chemical induced gastric mucosal injuries, such as monochloramine (Oyake et al., 2006), ethanol (Park et al., 2008). Others suggested that ginger also has a gastric protected activity by down regulation of the ulcer associated Bax protein with corresponding over expression of the HSP70 protein (AlRashdi et al., 2012). The expressed HSP70 interacts by proapoptotic Bax genes resulting in suppression of Bax activation in cells with high HSP-70 levels (Stankiewicz et al., 2005). The increased HSP-70 expression leads to an anti-apoptotic effect through the inhibition of Bax production (Rozza et al., 2014). Besides the enhancement of gastric mucus secretion and anti-oxidative capacity, ginger modulates inflammatory cytokine-mediated oxidative damage to the gastric mucosa (Ko and Leung, 2010), which may explain the reduction of the inflammatory cell infiltrates in group IV. Over expression of HSP70 and suppressed expression of Bax (AlRashdi et al., 2012) and iNOS (Hauser et al., 1996) proteins could have a protective role in gastric mucosal damage.

The present study is the first to investigate the protective effect of ginger on aspirin induced gastric ulcer on an extensive histological and immunohisto-chemical basis; however it has some limitations, such as it did not assess the volume of gastric juice and acid production induced by aspirin. This is because it was reported that gastric juice volume is not a major factor in ulcer formation or the protective effects of ginger powder seen in the experimental ulcer model rats (Wang et al., 2011). It is recommended to investigate the mechanism by which ginger increases HSP70 and decreases the iNOS and Bax proteins production which may be the key to its gastric protective effect. It is also recommended to compare the effectiveness of ginger and other pharmaceuticals currently used for the treatment of gastric ulcers.

In conclusion, the present study provided evidence that ginger protected against aspirin induced gastric ulcer. Ginger reduced the ulcer areas in the gastric wall and reduced or inhibited the edema and leucocyte infiltration of submucosal layers by upregulation of HSP70 and downregulation of iNOS and Bax proteins. Further studies are required to determine the bioactive components responsible for the mechanism of antiulcer activities of ginger indicated in this study.

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