Original Research

Nullification of aspirin induced gastrotoxicity and hepatotoxicity by prior administration of wheat germ oil in Mus musculus: histopathological, ultrastructural and molecular studies

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Abstract: Aspirin (acetyl salicylic acid) is used worldwide to treat various inflammatory conditions and prevent cardiovascular disease, along with reducing the risk of cancer. However, administration of aspirin causes toxic effects, especially in the stomach and liver. Thus, our study examined the protective effect of wheat germ oil on aspirin-induced toxicity in the stomach and liver tissues of Swiss albino mice. Administration of wheat germ oil before aspirin has restored normal hepatic and gastric tissue architecture and DNA integrity has become better than that of a negative health control group compared with the aspirin only treated group. The elevated gastric nitric oxide content in the aspirin only treated group was significantly decreased by wheat germ oil prior administration as a result of reduced the expression of inducible nitric synthase and increased the expression of endothelial nitric oxide synthase compared to their expression in the aspirin administered group. Wheat germ oil pre-administration significantly reduced the level of malondialdehyde, increased the level of glutathione and catalase and superoxide dismutase activities compared with those in aspirin only treated group. We conclude that wheat germ oil has a potential protective effect against aspirin induced gastro- and hepato-toxicity because of its free radical scavenging ability.

Key words: Aspirin gastro-toxicity; Hepatotoxicity; Wheat germ oil; Oxidative DNA damage; Gastric nitric oxide.

Introduction

Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug that is highly consumed in the world for the treatment of pain, fever, osteoarthritis and rheumatoid arthritis, and for the prevention of cardiovascular thrombotic diseases (1-3). In addition to its short intake after a heart attack to reduce the risk of death and long term use in high risk people to prevent heart attacks, strokes, and blood clots along with its ability to reduce the overall risk of both getting cancer and dying from cancer in particular colorectal cancer, it also reduces the risk of uterine, breast and prostate cancer (4-8). Furthermore, the Vad et al., (9) demonstrated the efficacy of acetylsalicylic acid as an anti-melanoma agent in B16-F0 cells and skin B16-F0 melanoma tumor mouse model.

However, the use of aspirin causes adverse effects on most organs leading to morbidity and mortality (10). Long-term therapeutic consumption of aspirin was associated with gastrointestinal ulcers, renal toxicity, liver toxicity and renal carcinoma (10, 11). The main problem with the use of aspirin is gastric ulcer (12). Similarly, Sostres *et al.,* (13) reported that aspirin use was associated with gastrointestinal side effectssuch as mucous ulcers, bleeding and perforation.

Generating free radicals is an acceptable mechanism for aspirin induced gastrostoxicity and hepatotoxicity as over-production of free radicals exhausted an antioxi-

dant defense system that has become unable to detoxify intermediate reaction causing oxidation and disrupt cellular integrity. Free radicals attack lipid membrane, protein and even DNA causing destruction of cell components and production of lipid peroxides (14, 15).

At present there is an increasing awareness of the importance of vegetable oils as sources of food and vehicles promoting health (16). Plant-derived compounds have the properties of disease prevention and potential health enhancing effects, so there is increasing interest in the use of these compounds as natural strong protective agents (17-18). One of the recent attractive plant derivatives is wheat germ oil for its powerful antioxidant and anti-inflammatory properties. Wheat germ oil is extracted from the wheat germ, a highest nutritious portion of the wheat kernel (19).

Wheat germ oil is an ideal source for toco-pherols, toco-trienols and phenol compounds with strong anti-inflammatory and antioxidant effects as evidenced by the low O^{2-} production and the activity of NADPH oxidase (20-23). It is also a good source of vitamin E which prevents oxidation processes in the body tissues and is rich in unsaturated fatty acids, especially linoleic and linolenic acids (precursors of Omega 6 and omega-3 fatty acids) (24-25).

Saleh et al., (26) reported that animal supplementation with the experimental diet containing 10% wheat germ oil caused an improvement in liver lesions caused by benzene injection for 9 weeks. Similarly, Sliai, (27)

revealed a decrease in caspase 3 immuno-reactivity and apoptosis, as well as an improvement in the hepatotoxic effect inducedby doxorubicin in animals orally administrated wheat germ oil (100 mg/kg b.w) with doxorubicinfor seven consecutive days.

Therefore, the study examined the potential protective effect of wheat germ oil against gastro- and hepatotoxicity of aspirin in Swiss albino mice using histological, ultrastructural and molecular studies.

Materials and Methods

Experimental animals

Thirty Swiss albino mice with body weights of 25-30gm have been used in this study.They were obtained from the animal house of National Organization for Drug Control and Research (NODCAR) and cared in accordance with the Guidelines of the National Institute of Health (NIH) regarding the care and use of animals for experimental procedures. They were housed under controlled condition of temperature 25°Cfeeding with standard chow diet (Protein: 160, 4; Fat: 36.3; fiber: 41g/kg and metabolizable energy 12.09 MJ)and had free access to drink water andwere deprived of food in mesh bottomed cages for 24 h prior to the beginning of experiment to diminish coprophagia but allow free access to water except for the last hour before the experiment according to protocol of Pan et al., (28).

Chemicals

Acetyl salicylic acid was obtained from Egyptian company for Chemical & Pharmaceuticals S.A.E., originally from China as a white powder with standard pharmaceutical grade, and was dissolved in distilled water justpriorits use to prepare the administration dose of acetyl salicylic acid which is 200mg/kg body weight, according toBukhari, et al., (29). The Omeprazole was purchased from Sigma-Aldrich Company(U.S.A) in the form of white powder with secondary standard pharmaceutical grade while wheat germ oil was obtained from El Captain Company (Cap Pharm) in Egypt.The doses used were 20 mg/kg for Omeprazole (30) and 1.5 ml/kg (~about 1400 mg/kg for wheat germ oil (31).

Experimental designs

After adaption for two weeks, thirty male Swiss albino mice were randomly divided into six groups (5 animals for each) and orally administered the tested substance using stomach tube once daily for three consecutive days as follow: negative control group was administered 1ml distilled water (group 1), while groups 2 and 3 were administered wheat germ oil at the dose level 1.5 ml/kg andOmeprazole at the dose level 20 mg/kg, respectively. The last three groups were administered aspirin at the dose level 200 mg/kg/kg body either alone (group 4) or one hour after omeprazole (group 5) or wheat germ oil (group 6) administration.One hour after the last administration at 3rd day, all animals sacrificed to expose stomach and liver tissues for macro/microscopically and ultrastructure examination and molecular studies. Small pieces of stomach were washed with cold PBS and immediately immersed in *RNA later* stabilized solution for RNA and DNA and stored at -80ºC until used.

Histological studies
Macroscopicexamination of gastric ulceration

The tissues of the stomach were opened along the greater curvature, then rinsed with saline to remove the attached debris and photographed the internal surface to record the hemorrhagic and ulceration areas.

Microscopic histological examination

Immediately after the gross lesions examination, part of the gastric and liver tissues of all groups were collected and fixed in 10% formalin for 48hr for histological examination. The samples were then washed, dried in an ascending chain of alcohol, cleared in xylene, embedded in paraffin wax and cut into five microns thick section using a microtome. These sections were then mounted on clean glass slides, stained with Ehrlich's haematoxylin-eosin (32) and examined under an Olympus microscope (BX41, Hamburg, Germany).

Ultrastructure examination

The ultrastructure was examined forgastric tissues only using electron microscope, and gastric samples (approximately 1 mm³, each) were immediately initial-fixed by immersion in 5% glutaraldehyde for 4 hours at room temperature. Secondary fixation carried out by immersion1 mm³ pieces of samples in 1% osmium tetroxide for 2 hours. Then dehydrated through ascending ethanol series, 30%, 50%, 70%, 90% and finally absolute ethanolfor 15 minutes at room temperature for each concentration.Absolute alcohol was replaced by propylene oxide or acetone a graduated series of ethanol: propylene oxide 2:1, 1:1 and 1:2, and then finally maintained in pure propylene oxide. The dehydrated specimens were embedded in an epoxy resin. Glass knives were used to obtain semi-thin sections (1 µm thick). These semi-thin sections (1 µm thick) were stained with toluidine blue to examine by light microscope. Ultra-thin sections (50-80 nm thick) of selected areas were contrasted with uranyl acetate and lead citrate and photographed with a JEOL 1010 Transmission Electron Microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Molecular studies
Gastric DNA breaks measurement

Both of single and double strand DNA breaks were measured in gastric individual cells using comet assay according to Tice et al. (33) method. After homogenized mall piece of gastric tissues in cold Hank's Balanced Salt Solutions without Ca^{++}& Mg^{++}, mixing 10 µl of clear cell suspension (≈10000 cells) with 75 µl of 0.5% low melting point agarose (Sigma) then spread them on a fully frosted slide covered with normal melting agarose (1%) and left to dry.Placed slides in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mMTris, pH 10, and freshly added 10% DMSO and 1% Triton X-100) for 24 hours at 4ºC in darkness. Then electrophoresed the unwinding DNA for 30 minute at 300 mA and 25 V (0.90 V⁄cm)in the Comet assay tank (LENNOX Laboratory Supplies, Cleaver Scientific Brand) after incubating the slides in the same fresh alkaline buffer (300 mMNaOH and 1 mM EDTA, pH>13). Finally neutralize the alkali by immersing the slides in 0.4 M Trizma base (pH 7.5) and fixed in 100% cold ethanol for

Table 1. Sequences of the used primers in quantitative RT-PCR amplification of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) genes compared to β-actin gene.

Gene	Sequences
iNOS	For: 5'-CGG GCA TTG CTC CCT TCC GAA AT-3'
	Rev: 5'-CTT CAT GAT AAC GTT TCT GGC TCT-3'
eNOS	For: 5'-TTC CGG CTG CCA CCT GAT CCTAA-3'
	Rev: 5'-AAC ATA TGT CCT TGC TCA AGGCA-3'
β-actin	For: 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3'
	Rev. 5'-GGA TGC CAC AGG ATT CCA TAC CCA-3'

permanent preparation. For scoring slides were stained with ethidium bromide (20 µg/ml) and then examined by epifluorescence microscope (Zeiss Epi-fluorescent) at 200X magnification and imaged the well spread cells. Fifty cells were scored using COMETSCORE software (Olive and Bananth, 1993) to analyze DNA damage using tail length in pixels (px), %DNA in tail and tail moment as end points.

Gastric and hepatic DNA fragmentation assessment

The effect of tested substances on integrity of DNA in the gastric and hepatic tissues was examined qualitatively by laddered DNA fragmentation assay using the method described by Sriram et al. (34).In brief: homogenized small portion of tissue in Tris EDTA (TE) lysis buffer with 0.5% sodium dodecyl sulfate then added RNase A (0.5 mg/mL) and incubated for 1 hour at 37ºC.Proteinase K (0.2 mg/mL) was finally added and incubated the sample at 50ºC overnight. The extracted DNA using Phenol extraction method was precipitated by ammonium acetate (7.5 M) and isopropanol and electrophoresed in 1% agarose gel for 45 minute at 70 V then visualized using a UV transiluminator and photographed.

Expression of gastric iNOS and eNOS genes

To measure the mRNA expression of the endothelial and inducible nitric oxide synthase; eNOS and iNOS respectively, extracted the total gastric RNA using Gene JET RNA Purification Kit (Thermo scientific, USA) and added DNase I (Thermo scientific, USA) to remove any genomic DNA remains. The purity and concentration of extracted RNA were determined by measuring the absorbance spectrophotometrically at 260 and 280 nm wavelength using Nanodrop device. Complementary DNA (cDNA) was synthesized from the purified total RNA using Revert Aid First StrandcDNA Synthesis Kit (Thermo scientific, USA) according to the manufacturer's instructions.

Expression of both eNOS and iNOS genes was measured quantitatively by SYBR green-based real time polymerase chain reaction (RT-PCR) using the 7500 Fast system (Applied Biosystem 7500, Clinilab, Egypt). The total PCR volume was 20 µl for each reaction and cycling was started with an initial denaturation at 95ºC for 15 min followed by thirty five cycles of denaturation at 95ºC for 15 s, annealing at 55 for 30s and extension at 72ºC for 1 min using the previously designed primers sequences (35-36)shown in table 1. Results of eNOS and iNOS expression were normalized against β actin gene as a reference gene and their expressions were

quantified using the comparative Ct (DDCt) method. Results were expressed as mean ± S.D of fold change in eNOS and iNOS expression compared to the untreated control level.

Gastric nitric oxide measurement

The concentration of nitric oxide (NO) was measured in the gastric tissues of all groups in accordance with the method described by Montgometry and Dymock (37) that depending on measuring the absorbance of the resulting bright reddish purple color of the azo dye at 540 nm using spectrophotometry.

Gastric lipid peroxidation and antioxidants estimation

The induction of lipid peroxidation was estimated by measuring the level of malondialdehyde (MDA) as a strong indicator of lipid peroxidation using the thiobarbituric acid (TBA) reaction with MDA in acidic medium at temperature of 95°C for 30 min and measuring the absorbance of the resultant pink spectrophotometrically at 534 nm (38).Antioxidants have been estimated by measuring the level of reduced glutathione (GSH) and enzymatic activities of catalase (CAT) and superoxide dismutase (SOD) as exactly as described by Beutler et al. (39), Aebi (40)and Nishikimi et al. (41) methods, respectively. Results were expressed as mean ± SD.

Statistical analysis

All results were shown as mean ± SD and analyzed using the Statistical Package for the Social Sciences (SPSS) (version 20) at the significant level <0.05. An independent sample t-test was used to estimate the effect of aspirin, Omeprazole or wheat germ oil on the tested parametersby comparing each group separately with the negative control group or with the similar positive control group (aspirin treated group) in the two groups' comparisons.

Results

Histological studies
Macroscopic examination of stomach tissues

As shown in Fig. 1 macroscopy of gastric tissues of mice treated with aspirin only showed large areas of sever hemorrhagic and marked ulcer lesions (Fig. 1B) compared to the normal appearance of the normal group (Fig. 1A). In contrast, treatment with omeprazole showed absence of visible hemorrhagic and ulcerative sites (Fig. 1C), as well as pretreatment with omeprazole prior aspirin administration clearly alleviated ulcera-
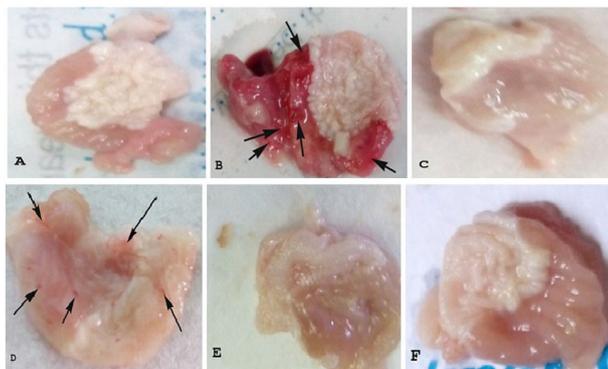
Figure 1. Macroscopic examination of the stomach tissues from: (A) control group ;(B) Aspirin treated group; (C) Omeprazole treated group; (D) Omeprazole-aspirin treated group; (E) Wheat germ oil treated group and (F) Wheat germ oil-aspirin treated group. Arrow: hemorrhagic and ulceration lesions.

tive lesions caused by aspirin, and showed only a few hemorrhagic lesions compared to the treated group with aspirin (Fig. 1D). Furthermore, no signs of ulceration were observed in the gastric tissues of mice administered only wheat germ oil (Fig. 1E) and treatment with wheat germ oil before aspirin revealed significant improvement as revealed by the absence of visible hemorrhagic and ulcerative sites observed in mice treated with aspirin (Fig. 1F).

Microscopically examination of:
Stomach tissues

Histological examination of the stomach tissues of control animals revealed normal appearance of the mucosa and sub-mucosa layers (Fig.2A). However, ulceration and distortion of gastric glands were observed along with edema and inflammatory cells infiltration in the mucosa layers in a large area of the gastric section of mice treated with aspirin only as well as muscle was broken into the gastric sub-mucosa layers (Fig. 2B1) in addition to the scattered severe erosion of mucosa (Fig.
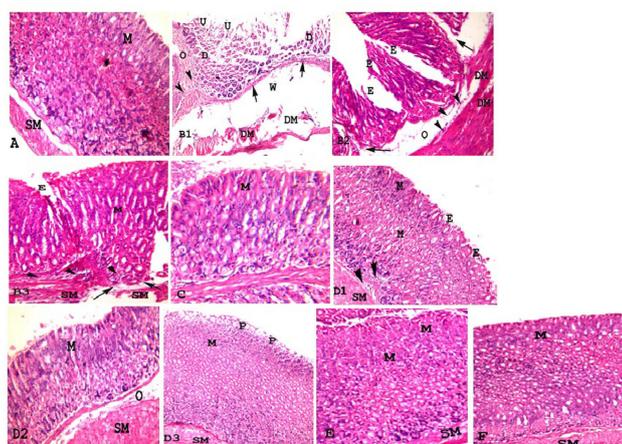

Figure 2. A photomicrograph of stomach tissues from: (A) control group ;(B1-B3) Aspirin treated group; (C) Omeprazole treated group; (D1-D3) Omeprazole-aspirin treated group; (E) Wheat germ oil treated group and (F) Wheat germ oil-aspirin treated group. (H & E., X 200). Mucosa layer (M); ulceration area (U); Degenerative of gastric glands (D); Degenerativesuperficial layers (P); Erosion of mucosa (E); Destroy muscle of mucosa layer (arrow); edema (O); inflammatory cells infiltration (arrow head); Widening submucosa (W); Destruction muscular layer of sub-mucosa (DM).

2B2) and dense inflammatory cells aggregation in submucosa layers (Fig. 2B3).

No histologic changes were observed in both the gastric mucosa and sub-mucosa of mice administered only Omeprazole (Fig. 2C) in the opposite direction to moderate diffuse erosion observed in the mucosa, minor edema and inflammatory cell aggregation observed in the sub-mucosa layer coupled with moderate degenerated superficial layers despite the normal appearance of gastric mucosa and sub-mucosal layers of pretreated mice with Omeprazole before administration of aspirin (Fig. 2D1, D2 & D3). Indeed, the gastric sections of Swiss albino mice treated only with wheat germ oil showed no signs of histological injuries in both mucosa and sub-mucosal layers as well as those of mice treated with wheat germ oil prior aspirin administration revealed a marked improvement and restoration of the normal structures of both the mucosa and sub-mucosa layers (Fig 2E & 2F).

Liver tissues

The microscopic examination of the liver sections of the negative control mice revealed the normal architecture of liver tissues as liver cells are arranged in the radiated cords of the central veins. The narrow spaces between the hepatic cords formed the hepatic sinuses that Kupffer cells had designated. Polygonal hepatic cells possess acidophilic cytoplasm and dark stained nuclei. At the angles of the lobules, the portal areas are found, that contains portal vein, bile duct, and artery ensheathed by connective tissue (Fig. 3A). Conversely, a section of the liver of the animals treated with aspirin only showed severe vacuolated hepatocyte, markedly scattered dilated congested central vein and dilated congested sinusoid compared with control group. The dense aggregations of inflammatory cells in hepatic tissues around the blood vessels (central vein and portal vein) and loss of sinusoids and hepatocytes with pyknotic nuclei (Fig. 3B1 & 2) were observed in addition to the enlarged portal areas observed with marked expansion of the congested portal vein,and many dilated bile ducts in other regions besides necrotic areas, hemorrhage and aggregation of inflammatory cells infiltrating hepatic tissues (Fig. 3B2&B3).

Livers of mice administered only Omeprazole showed some areas with normal tissue structure, intact liver cells, central vein, with mild vacuolation of some hepatic cells(Fig. 3C1)whilethe central vein was observed to be moderately enlarged andin the other areathe liver cells were vacuolated and Kupffer cells were mild activated (Fig. 3C2). The same is true for pretreated mice with omeprazole prior aspirin revealed the normal appearance of liver cells, densely dispersed aggregations of inflammatory cells around marked congested dilated central vein(Fig. 3D1). In addition, there are other regions with many congested central veins, and hepatocytes either with severe vacuolation or pyknotic nuclei (Fig. 3D2). Mostof the portal areas have been observed to have sever congested dilated portal vein, proliferated dilated bile duct, moderate inflammatory cells aggregation (Fig. 3D3) and scattered moderate inflammatory cells aggregations (Fig. 3D4). On contrary, no signs of hepatic injuries were observed in the liver sections of mice that were administered either wheat germ
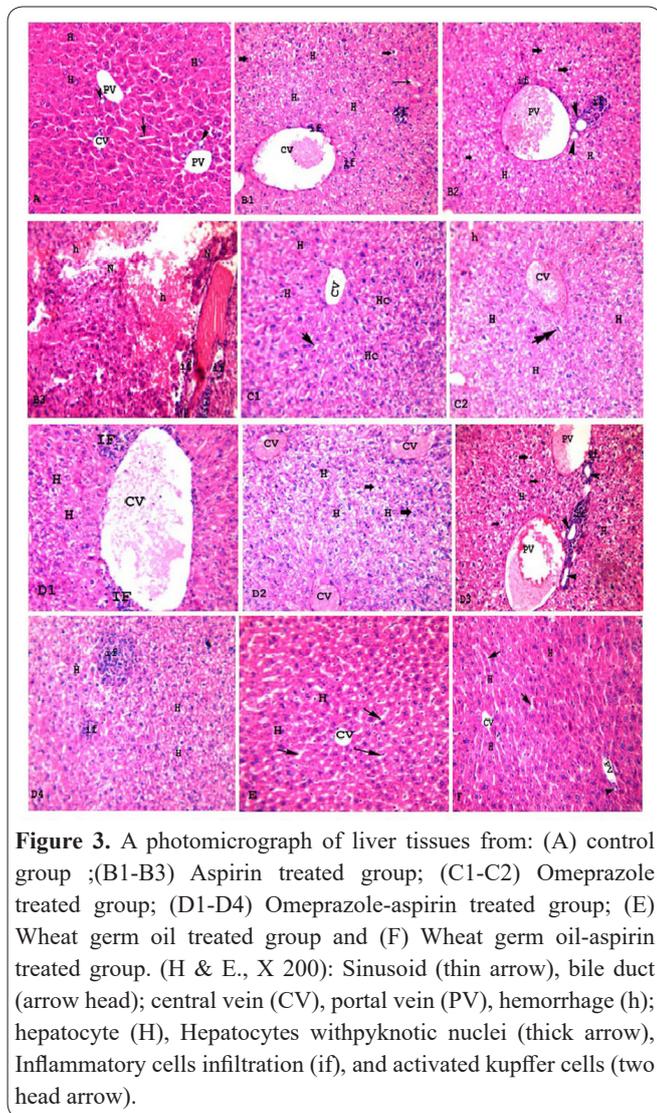
Figure 3. A photomicrograph of liver tissues from: (A) control group ;(B1-B3) Aspirin treated group; (C1-C2) Omeprazole treated group; (D1-D4) Omeprazole-aspirin treated group; (E) Wheat germ oil treated group and (F) Wheat germ oil-aspirin treated group. (H & E., X 200): Sinusoid (thin arrow), bile duct (arrow head); central vein (CV), portal vein (PV), hemorrhage (h); hepatocyte (H), Hepatocytes withpyknotic nuclei (thick arrow), Inflammatory cells infiltration (if), and activated kupffer cells (two head arrow).

oil only (Fig. 3E) or prior to administration of aspirin that shown normal hepatic architecture and restoration ofthe normal hepatocyte, central vein, sinusoid despite aspirin administration (Fig. 3F).

Ultrastructure examination of stomach
Neck mucosa cells

Electron microscopic examination of mucosal neck cells of gastric tissue from normal animals revealed low columnar cells with many apical secretory granules, normal basal nucleus and rough endoplasmic reticulum (Fig. 4a). While, irregular indented basal nucleus, degenerated mitochondria and dilated rough endoplasmic reticulum were seen in animals treated with aspirin. In addition to, mucous granules were appeared as coalescence(Fig. 4b). The gastric tissue of pre-treated Swiss albino mice with omeprazole before aspirin showed mucus cells of the neck almost intact but with irregular base nucleus and moderate degeneration in some secretory granules compared to the control group (Fig. 4c). furthermore,the intact structure of the neck mucous cells were restored in the gastric tissues of pre-treated mice with wheat germ oil prior to aspirin administration as shown by the observed vesicular nucleus and normal appearance of apical granules compared to the aspirin treated group and control group (Fig. 4d).
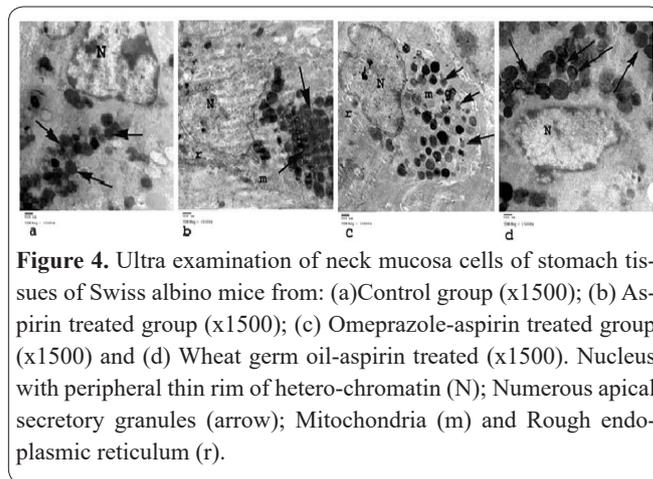
Parietal cells

Figure 4. Ultra examination of neck mucosa cells of stomach tissues of Swiss albino mice from: (a)Control group (x1500); (b) Aspirin treated group (x1500); (c) Omeprazole-aspirin treated group (x1500) and (d) Wheat germ oil-aspirin treated (x1500). Nucleus with peripheral thin rim of hetero-chromatin (N); Numerous apical secretory granules (arrow); Mitochondria (m) and Rough endoplasmic reticulum (r).

The electron microscopy of the parietal cells of the control animals showed large pyramidal cells with central rounded, vesicular nuclei (euchromatic), numerous intracellular canaliculi and electron dense mitochondria distributed in the their cytoplasm (Fig. 5A). On the other hand, animals treated only with aspirin revealed highly irregular shrunken indented nucleus with scattered numerous dilated intracellular canaliculi (Fig. 5B1). Some parietal cells revealed either pyknotic nucleus or degenerated nucleus along with degraded mitochondria, and excessive expansion of intracellular canaliculi compared to control group (Fig. 5B2).

When animals were pretreated with omeprazole prior to aspirin administration the normal appearance of the nucleus, with slight dilation of intracellular canaliculi in most parietal cells were observed compared with the control group. Most of the mitochondria had a normal appearance and had few irregular shapes (Fig. 5C). While animals previously treated with wheat germ oil showed no changes in the ultra-structures and normal reappearance of the nucleus and mitochondria compared to the control group and the aspirin only treated group (Fig. 5D).

Cells which contact gland lumen

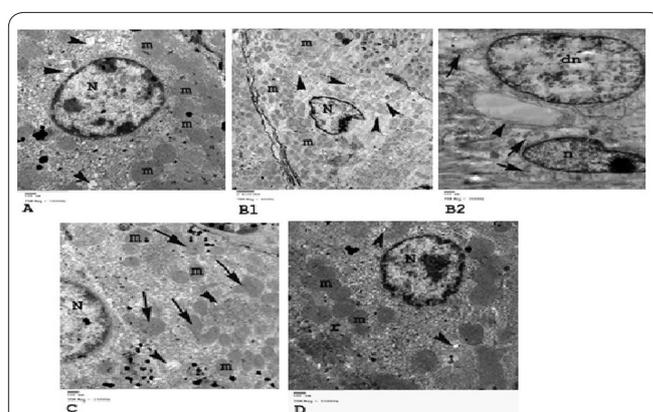Ultra-thin sections of the gland lumen cells contact

Figure 5. ultra-examination of parietal cell from stomach glands of Swiss albino mice. (A)Control group (x15000); (B1-B2) Aspirin treated group (x6000; x15000); (C) Omeprazole-aspirin treated group (x15000) and (D) Wheat germ oil -aspirin treated group (x15000).Nucleus with peripheral thin rim of hetero-chromatin (N); pyknotic nucleus (n); distorted nucleus (dN); Mitochondria (m); degenerated disrupted mitochondria with irregular shape (arrow); Intracellular canaliculi (arrow head).
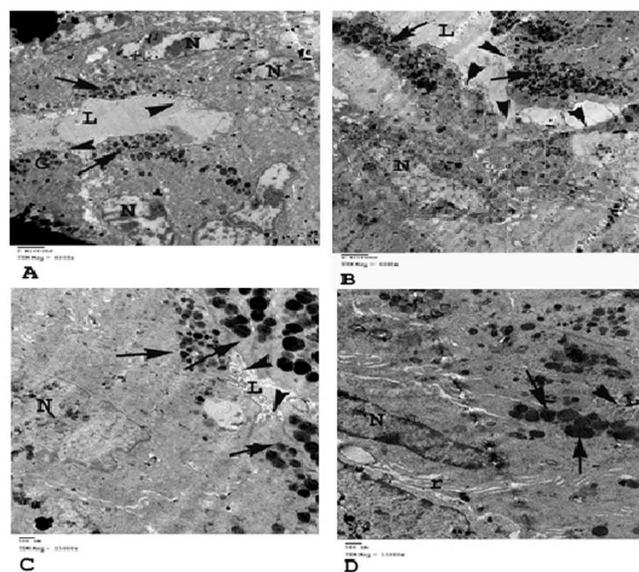
Figure 6. ultra-examination ofcells which contact lumen of glands of stomach tissues of swiss albino mice from: (A)control group (x6000); (B) aspirin treated group (x6000); (C) omeprazole-aspirin treated group (x15000) and (D) wheat germ oil-aspirin treated (x1500). Gland lumen (L); apical surface cell with microvilli (arrow head); nucleus (n); apical secretory granules (arrow); and rough endoplasmic reticulum (r).

nucleus and rough endoplasmic reticulum compared to the aspirin only treated group (Fig. 6D).

Molecular studies

Gastric DNA breaks

The induction of both single and double DNA breaks was assessed using the alkaline comet assay in the gastric tissues of all groups.The results were summarized in Table 2.The oral intake of either Omeprazole (20 mg/kg) or wheat germ oil (1.5 ml/kg) alone for three consecutive days did not cause any significant changes in DNA breaks estimated parameters tail length, %DNA in tail and tail moment ($p>0.05$) compared with the negative control levels. On contrary, aspirin (200 mg/kg) administrated alone for three consecutive days induced intensive DNA breaks as shown by the significant elevations in tail length ($p<0.01$), %DNA in tail ($p<0.05$) and tail moment ($p<0.01$) compared to negative control levels (Table 2).

Prior administration of either Omeprazole (20 mg/kg) or wheat germ oil (1.5 ml/kg) one hour before aspirin (200 mg/kg) oral administration significantly reduced the DNA breaks caused by aspirin as revealed by the observed significant marked reduction in tail length, %DNA in tail and tail moment compared with those in aspirin only treated group and not only reached the negative control levels but became significantly lower than the negative control levels in the group treated with wheat germ oil prior to aspirin (Table 2).

Gastric and hepatic DNA fragmentation

Gastric and hepatic DNA fragmentation has been estimated using a pulsed gel electrophoresis for the genomic DNA of all groups and the running patterns of genomic DNA were shown in Fig. 7. Oral administration of aspirin (200 mg/kg) alone for three consecutive days caused fragmentation of both gastric and hepatic DNA as revealed by its fragmented appearance compared to the normal intact DNA of the negative control group.

However, pre- administration of Omeprazole (20 mg/kg) or wheat germ oil (1.5 ml/kg) before the administration of aspirin declined the aspirin induced DNA fragmentation and restored the normal intact pattern of genomic DNA running on the agarose gel in gastric and liver tissues (Fig. 7). Furthermore,the pattern of gastric and hepatic genomic DNA of mice treated with either Omeprazole or wheat germ oil was identical to that of

of the negative control group revealed columnar cells with intact apical surface and normal long apical microvilli.Also apical numerous secretory granules and vesicular nucleus with peripheral thin rim of hetero-chromatinwere noticed (Fig. 6A). However, those of animals only treated with aspirin exhibited severe damage as demonstrated by markedlydeformed cell membrane of cells with disrupted apical microvilli and irregular degenerated nucleus as well as the most granules were degenerated and aggregated at the apical surface of cells compared with the control group (Fig. 6B).

Animals previously treated with omeprazole prior to aspirin showed intact cells but with moderate degenerated microvilli in the apical surface, the irregular nucleus and most granules were accumulated inthe apical surface of the cells compared to aspirin only treated and control groups (Fig. 6C). In addition, pretreated animals with wheat germ oil prior to aspirin administration revealed the restoration of the normal apical cell membrane with intact microvilli, along with the vesicular

Table 2. levels of DNA breaks in gastric tissue of mice administered aspirin (200 mg/kg) (Asp), wheat germ oil (1.5 ml/kg) (WGO) or/and Omeprazole (20 mg/kg) (Omp) for three consecutive days.

Treatment	Tail length (px)	%DNA in tail	Tail moment
Negative control	6.34 ± 2.27	31.29 ± 7.85	2.58 ± 1.27
WGO	5.52 ± 1.65	35.44 ± 2.31	3.03 ± 0.45
Omp	4.61 ± 1.16	24.18 ± 6.43	1.69 ± 0.77
Asp	$11.86 \pm 1.81^{a**}$	$42.60 \pm 5.54^{a*}$	$5.56 \pm 0.71^{a**}$
WGO prior Asp	$3.56 \pm 1.16^{a*, b***}$	$16.09 \pm 3.55^{a**,b***}$	$0.66 \pm 0.34^{a*, b***}$
Omp prior Asp	$5.82 \pm 2.69^{b**}$	$24.44 \pm 11.97^{b*}$	$1.53 \pm 0.95^{b***}$

Results are expressed as mean ± SD. using independent t-test each group of the five treated groups has been compared separately with the negative control group and also each of the last two groups has been compared separately with the aspirin treated group. [a]: indicating significant difference from the negative control group and [b]: indicating significant difference from the aspirin treated group at [*]: $p<0.05$, [**]: $p<0.01$ and [***]: $p<0.001$.
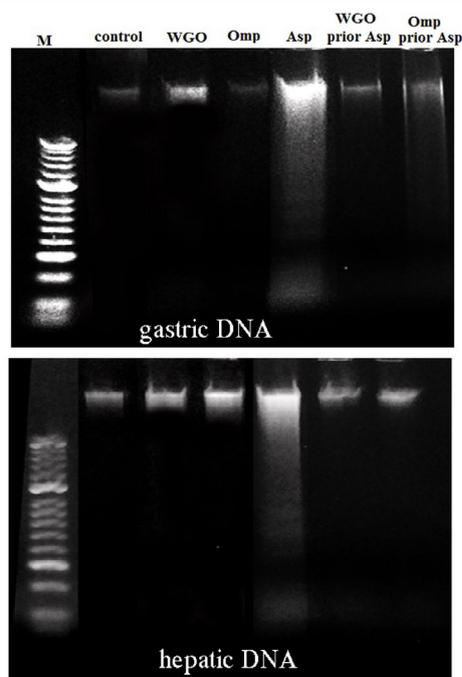
Figure 7. Pattern of gastric and hepatic genomic DNA of mice administered aspirin (200 mg/kg) (Asp), wheat germ oil (1.5 ml/kg) (WGO) or/and Omeprazole (20 mg/kg) (Omp) for three consecutive days running on agarose 1.5% and stained with ethidium bromide. M: marker.

the negative control group as shown in Fig. 7.

Expression of gastric iNOS and eNOS genes

Results of RT-PCR showed that the expression of either iNOS or eNOS did not changed and remained in the control level in groups administered either wheat germ oil (1.5 ml/kg) or Omeprazole (20 mg/kg) alone. Although administration of aspirin (200 mg/kg) led to statistical significant increases (p<0.001) in the expression of iNOS and decreases ((p<0.001) in the eNOS expression compared to the negative control level as shown in Fig. 8, Omeprazole or wheat germ oil administration prior to aspirin significantly altered the expression of iNOS and eNOS genes (p<0.001) compared to the aspirin alone treated group and their expression levels not only reached the control level but also became much better expressed as shown in the expression level of eNOS gene (p<0.01) in group pre-administered wheat germ oil before aspirin.

Gastric nitric oxide

Measuring the contents of nitric oxide (NO) in gastric tissues showed that oral administration of aspirin resulted in statistical significant increases (p<0.001) in gastric NO content compared with negative control level. Conversely, NO content was significantly decreased (p<0.001) in the groups previously administered Omeprazole (20 mg/kg) or wheat germ oil (1.5 ml/kg) before administration of aspirin compared to the aspirin alone administered group (Fig. 9). In addition, there was no significant change in NO content after Omeprazole administration while wheat germ oil administration significantly decreased (p<0.05) the gastric NO content compared with the negative control level (Fig. 9).
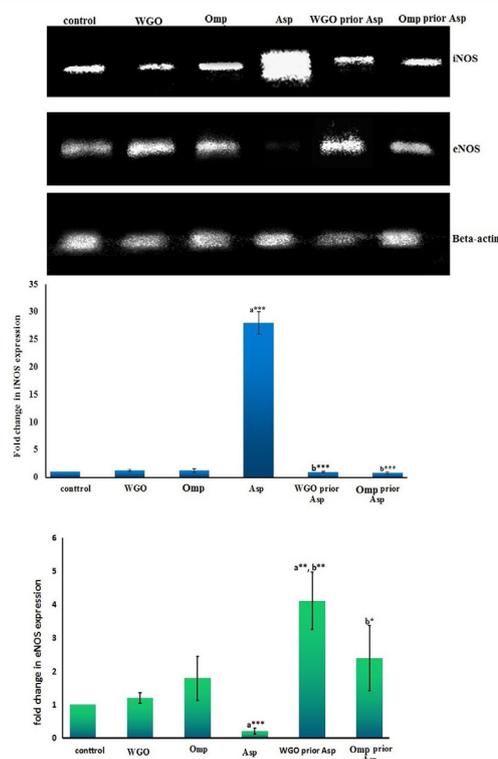
Gastric lipid peroxidation and antioxidants

Figure 8. Expression level of gastric inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) compared to the reference gene beta actin gene in mice administered aspirin (200 mg/kg) (Asp), wheat germ oil (1.5 ml/kg) (WGO) or/and Omeprazole (20 mg/kg) (Omp) for three consecutive days. Results are expressed as mean ± SD. a: indicating significant difference from the negative control group and b: indicating significant difference from the aspirin treated group at *: p<0.05, **: p<0.01and ***: p<0.001 using independent t-test.
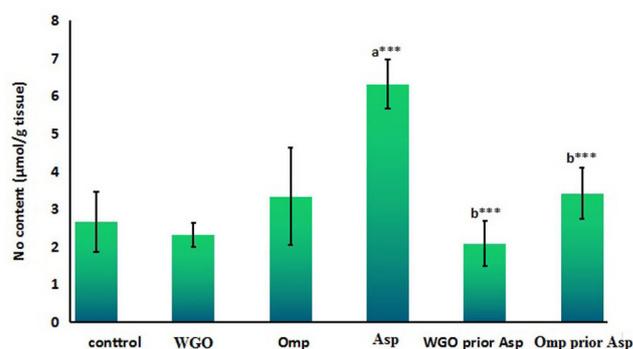

Figure 9. Nitric oxide (NO) content in the gastric tissue of mice administered aspirin (200 mg/kg) (Asp), wheat germ oil (1.5 ml/kg) (WGO) or/and Omeprazole (20 mg/kg) (Omp) for three consecutive days. Results are expressed as mean ± SD. a: indicating significant difference from the negative control group and b: indicating significant difference from the aspirin treated group at ***: p<0.001 using independent t-test.

The lipid peroxidation and antioxidant status in the stomach did not change in the groups orally administered either wheat germ oil ((1.5 ml/kg) or Omeprazole (20 mg/kg) alone as shown by insignificant changes in the level of MDA and GSH and the enzymatic activities of CAT and SOD (p>0.05) compared to negative control levels (Table 3).

In addition, the results showed that oral administration of aspirin (200 mg/kg) for three consecutive days significantlyincreased the level of MDA (p<0.001) and

Table 3. The levels of malondialdehyde (MDA) and reduced glutathione (GSH) and the enzymatic activities of catalase (CAT) and superoxide dismutase (SOD) in gastric tissue of mice administered aspirin (200 mg/kg) (Asp), wheat germ oil (1.5 ml/kg) (WGO) or/and Omeprazole (20 mg/kg) (Omp) for three consecutive days.

Treatment	MDA level (nmol/g tissue)	GSH level (mmol/g tissue)	CAT activity (U/g tissue)	SOD activity (U/g tissue)
Negative control	71.40 ± 10.92	5.70 ± 0.76	11.12 ± 1.61	21.49 ± 2.39
WGO	75.20 ± 8.34	6.38 ± 0.66	12.66 ± 0.64	23.14 ± 1.67
Omp	74.36 ± 4.44	5.54 ± 0.93	10.96 ± 1.21	21.18 ± 2.37
Asp	$158.51 \pm 7.98^{a***}$	$1.39 \pm 0.23^{a***}$	$4.17 \pm 0.84^{a***}$	$10.01 \pm 3.17^{a***}$
WGO prior Asp	$68.85 \pm 6.32^{a*, b***}$	$7.21 \pm 0.85^{a*, b***}$	$11.18 \pm 1.24^{a*, b***}$	$24.42 \pm 3.24^{b***}$
Omp prior Asp	$66.48 \pm 2.31^{b**}$	$4.32 \pm 0.84^{a*, b***}$	$9.68 \pm 0.86^{b***}$	$20.12 \pm 3.12^{b**}$

Results are expressed as mean ± SD. using independent t-test each group of the five treated groups has been compared separately with the negative control group and also each of the last two groups has been compared separately with the aspirin treated group. [a] indicating significant difference from the negative control group and [b] indicating significant difference from the aspirin treated group at [*] $p<0.05$, [**] $p<0.01$ and [***] $p<0.001$.

decreased the level of GSH($p<0.001$) and enzymatic activities of both CAT and SOD enzymes compared to negative control levels. On contrary, pre-administration of either Omeprazole or wheat germ oil resulted in statistical significant decreases in the MDA level ($p<0.001$) and significant increases in both the GSH level ($p<0.001$) and activities of CAT and SOD enzymes compared with aspirin alone treated group (Table 3).

Discussion

The focus on wheat germ oil has increased over the past few years due to its strong anti-oxidant and anti-inflammatory properties. Therefore, we evaluated the possible protective role of wheat germ oil on aspirin induced gastro- and hepato-toxicity in mice.

Although aspirin is a readily available cheap drug and is widely used as anti-inflammatory, anti-platelet, analgesic and antipyretic drug, its long term use threatens human life as a result of aspirin induced gastrointestinal ulcers, hepatotoxicity and nephrotoxicity that may extend to renal cell cancer(42-43). Histological data confirmed the previously reported aspirin induced gastro-toxicity and hepatotoxicity (44-45) even on fine structure through severe tissue lesions observed in both gastric and hepatic tissues of the aspirin administered group. Our findings from the dense aggregation of inflammatory cells revealed that these changes could be attributed to the enhanced reactive oxygen species (ROS) productionby the infiltrating inflammatory cells in the gastric and hepatic tissues of the aspirin administered group in accordance with the previous studies (14,15,46).

An increase in ROS production by the administration of aspirin was demonstrated in this study by significant elevations in the MDA levelthat exhausted the antioxidant defense system as shown by reduced level of cellular GSH and decreased activity of both CAT and SOD enzymes. Aspirin administration also increased the gastric NO contents by upregulation of the iNOS expression and downregulation of the eNOS expression and thus increased the reactive nitrogen species (RNS). Subsequently, the increased production of ROS and RNS disrupted the normal balance between oxidants and antioxidants in the cell causing oxidative stress (47-48). Highly reactive ROS and RNS can react and

damage macromolecules including lipid, carbohydrates, protein and even DNA via nitration and oxidation (49) so the DNA damage shown in the genomic DNA of both the gastric and hepatic tissues after aspirin administration was due to the increased ROS and RNS molecules.

ROS are one of the important factors in causing mucosal damage through oxidative damage in the cell membrane and intracellular molecules (46). Similarly, Pohle et al., (14) revealed that the gastric damage caused by aspirinin human is due tothe marked increase in the generation of free radicals that caused lipid peroxidation and suppressed antioxidant enzymes.At the same time high NO levels caused by high iNOS expression induced sever inflammation and oxidative damage as NO have the ability to interact with superoxide anion leading to the formation of peroxynitrite causing cytotoxicity, nitration, hydroxyl radical production and oxidative DNA damage(50-52).

On the contrary, NO synthesized by overexpression of eNOS maintains the integrity of the gastric epithelium, regulates the blood flow in gastric mucosa and leads to the secretion and synthesis of mucus in the stomach and thus accelerate the healing of gastric ulcer (53). Therefore, the observed recurrence of the normal hepatic and gastric architecture and normal DNA integrityafter pre-administration of wheat germ oil prior to administration of aspirin can be interpreted through themanifested over expression of eNOS and reduced expression of iNOS thus increasing the beneficial synthesis of non-toxic NO that promote gastric ulcer healingand decrease the synthesis of harmful toxic NO that caused oxidative damage.

As a result, the intake of wheat germ oil before the administration of aspirin reduced RNS as well as the generation of ROS resulting by aspirin by inhibition of the production of MDA and augmented antioxidant defense system as shown by the increased level of GSH and the enzymatic activities of CAT and superoxide dismutase compared to those found in aspirin treated group.Similar results from previous studies have shown that the antioxidant activity and free scavenger ability of wheat germ oil are due to its composition such as alpha-linolenic acid and vitamin E which have a strong antioxidant activity through the anti-inflammatory effect and limit the production of superoxide and the oxidase activity of nicotinamide adenine dinucleotide phosphate

(NADPH) in addition to inhibition of oxidation process (22, 54-56). Furthermore, the study of Mohamed and Ahmed, (57) revealed that oral supplementation with wheat germ oil (54mg/Kg body weight) for 14 successive days down regulatedexpression of pro-apoptotic genes in irradiated rats.

Similarly, normal gastric and hepatic architecture and DNA damage integrity were restored after Omeprazole prior administration due to the antioxidant potential of Omeprazole demonstrated by modulating the expression of both iNOS and eNOS genes in the manner that shifting NO synthesis in the useful healing NO direction and also by potentiating the antioxidant defensive system that depleted the harmful MDA and restored the GSH level and activities of CAT and SOD enzymes suppressed by aspirin administration in consistent with previous studies (58-60). However, few signs of gastric hemorrhagic lesions and hepatocellular lesions including congested enlarged portal vein, proliferated dilated bile ducts, and accumulation of inflammatory cells in most portal areas combined with necrotic areas were observed after Omeprazole administration either alone or before aspirin intake. On the other hand, no signs of toxicity were observed after the administrationof wheat germ oil in the architectures of gastric and hepatic tissues.

In conclusion prior administration of wheat germ oil has attenuated the toxicity of aspirin by modifying the expression of the iNOS and eNOS genes and thus shifted the NO synthesis in the non-toxic beneficial direction in addition to its ability to scavenger free radicalsshown by the decreased MDA level and increased antioxidants. Moreover, wheat germ oil exhibited a more powerful anti-ulcerability and safety than commercially used Omeprazole drug.

Acknowledgements

Conflict of interest
Authors declared no conflict of interest.

Author's contributions
Each author has the same efforts and contribution in this manuscript.

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