Histological Study of the Effect of Induced Cholestasis on Gastric Fundic Mucosa of Adult Male Albino Rats and the Role of Nigella Sativa (Black Seed) Supplementation

Magdy F. Gawish, Maha A. Khattab, Dalia A. Mohamed and Nahla E. Ibrahim
Department of Histology and Cell Biology, Faculty of Medicine, Zagazig University, Egypt.

*Corresponding author:
Dalia A. Mohamed

Histology and Cell Biology Department, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt.
Tel.: 00201223168887
E-mail: daliafarag@gmail.com

Abstract

Introduction: Cholestasis is an impairment of the bile flow. It could be results from either extra or intrahepatic causes. It leads to retention of the bile acids, bilirubin and other bile contents in the liver and blood. Elevated levels of bile acids in the liver may induce apoptosis or necrosis of hepatocytes and eventually cirrhosis. Liver cirrhosis (LC) is a dangerous public health problem. The stomach is one of the main targets of cholestatic liver diseases. Nigella Sativa (NS) or Black Seed (BS) extracts have shown several therapeutic effects including its antioxidant, hepatoprotective, immunomodulatory, anti-inflammatory and anti-tumor activities. Aim of the work: Detection of histological and immunohistochemical changes that may occur in the fundic mucosa of adult male albino rats after induction of cholestasis and the role of BS supplementation. Materials and methods: Sixty three adult healthy male albino rats were classified into three groups; control (Ia,Ib,Ic) , cholestatic-induced and BS-supplemented group.

At the end of the experiment, the stomach of all rats was processed for histological Hematoxylin and Eosin (H&E), PAS-alcian blue and immunohistochemical staining for detection of chromogranin-A , transmission and scanning electron microscope study. The obtained results were analyzed morphometrically and statistically. Results: The fundic mucosa of cholestatic-induced group showed variable degrees of fundic mucosal lesions as disorganized fundic glands and loss of its normal architecture. BS supplemented group revealed apparently normal architecture. Histomorphometric study and statistical analysis confirmed the results. Conclusion: cholestasis induced alteration in the fundic mucosa and its mucus barrier. Marked improvement with BS administration was detected. So, BS is recommended to protect the fundic mucosa against these changes in hepatic patients.

Keywords: gastric fundic mucosa, BS, cholestasis, transmission and scanning electron microscope.

INTRODUCTION

Cholestasis is an impairment of the bile flow from the liver to the duodenum. There are two main types of cholestasis; obstructive (extrahepatic) and metabolic (intrahepatic). Obstructive cholestasis is a mechanical blockage in the duct system while metabolic type usually occurs as a result of any disturbances in the bile formation (1).Obstructive (extrahepatic) cholestasis is a mechanical blockage of the common bile duct or Ampulla of Vater by bile stones, parasitic infestation, bile duct carcinoma and pancreatic head carcinoma. On the other hand, metabolic (intrahepatic) cholestasis may be caused by alcoholic liver disease, amyloidosis and lymphoma (2,3).
Several reports have pointed to the relation between cholestatic liver diseases and different body organs like kidney, heart, lung and brain. Moreover, it was revealed that cholestasis was associated with cardiovascular complications as hyperdynamic circulation and portal hypertension. Additionally, changes in blood volume distribution and the release of vasoactive substances may lead to cardiopulmonary manifestations as hepatopulmonary syndrome (HPS), portopulmonary hypertension (PPH) and cirrhotic cardiomyopathy (4). It was found that patients with hepatic diseases usually complain of gastrointestinal symptoms as abdominal pain, loss of appetite, vomiting and bleeding tendency. It was always thought that haematemesis and other gastrointestinal symptoms are related to esophageal varices, while other researchers claimed that these symptoms are due to affection of the stomach in hepatic patients(5).

Among various medicinal plants, Black Seed (BS) or Nigella Sativa (NS) is emerging as a wonderful herb with a rich historical and religious background since many researches revealed its wide spectrum of pharmacological potential. Various clinical and experimental studies have shown several therapeutic effects of BS extracts including its antioxidant, hepatoprotective, immunomodulatory, anti-inflammatory, anti-tumor, antidiabetic and analgesic activities. BS has been used for treatment of headache, toothache, nasal congestion, intestinal worms, conjunctivitis and abscesses. Today, it is used for treating digestive tract conditions including gases, colic, diarrhea, dysentery, constipation and hemorrhoids (6&7). Therefore, the goal of the present study was to investigate gastric lesions in adult male albino rats after induction of cholestasis and the possible protective role of BS supplementation.

MATERIALS AND METHODS

**Animals:** Sixty three healthy adult male albino rats (4-6 months) weighing 200-220gm were used in this study. The animals were obtained from the Animal House, Faculty of Medicine, Zagazig University, Zagazig, Egypt. They were fed standard balanced diet and allowed water ad-libitum. They were housed in hygienic cages in 12 h light/12 h dark cycle at room temperature according to the guidelines for animal research issued by the National Institute of Health and approved by Animal Ethics Committee, Zagazig University.

**Chemicals:**
1- Black seed (BS) was purchased from Kahira Pharm. and Chem. Ind. Co., Cairo, Egypt.

Soft gelatin capsules containing 500 mg of oil were used.

**Experimental procedure:**

**Group I (Control):**

Twenty one healthy adult male albino rats were equally subdivided into three subgroups

**Subgroup (Ia):** were not subjected to any procedure (non operated).

**Subgroup (Ib):** were subjected to sham operation.

**Subgroup (Ic):** sham operated + BS oil at a dose of 50 mg/kg bw daily for successive 30 days via gastric gavage. Surgical procedures were performed under anesthesia using 4 mg /100gm bw phenobarbital intraperitonealy. Rats’ abdomen was sterilized and midline laparotomy was done. Bile duct identification and manipulation without ligation were made.

**Group II (Cholestatic induced):** this group included twenty one healthy adult male albino rats. The abdomen was shaved, disinfected with 10% povidone iodine and midline laparotomy was performed. The common bile duct isolated and totally ligated with 4–0 silk suture for 30 days. The rats were allowed to recover with free access to chow and water (8).

**Group III (BS supplemented):** Rats of this group were subjected to the same surgical procedure, after one month the animals were received black seed oil at a dose of 50mg/kg bw daily for successive 30 days via gastric gavage. Soft gelatin capsules (500 mg) of BS oil were dissolved in 10 cm of vegetable oil. At the end of the experiment, the rats were fasted.
overnight. They were sacrificed with intraperitoneal injection of pentobarbitone sodium 60 mg/kg body weight (9) and their stomach were dissected out, rinsed and cut along the greater curvature. Specimens from the fundus region of stomach were prepared for light and scanning electron microscope examination. Also, specimens from their liver were processed for light microscopic examination to assess liver affection.

**Histological study:**
Specimens for light microscopic examination were fixed in 10% neutral formol saline, processed for paraffin block preparation, cut into 7 µm sections, and subjected to H&E, PAS-alcan blue histochemical method was used to differentiate neutral mucin from acidic mucin and immunohistochemical stain for detection of chromogranin A in enteroendocrine cells.

Immunohistochemical reaction was carried using avidin biotin peroxidase system. The primary antibody (chromogranin A antibody) used was a mouse monoclonal antibody of IgG immunoglobulin type (Dako Life Trade -Egypt, clone 1A4, code No. M0851). It was obtained from Sigma Biochemical (St. Louis, Missouri, USA). The antigen was finally localized by the addition of DAB Chromogen. Slides were counterstained with Mayer's haematoxylin (10).

**Transmission electron microscopy (TEM):**
The specimens were cut into small pieces of about 1mm3 size and rapidly fixed in 2.5% glutaraldehyde for 24h. Specimens were washed in 0.1mol/l phosphate buffer at 4°C and then postfixed in 1% osmium tetroxide at room temperature. Specimens were dehydrated in ascending grades of ethyl alcohol and embedded in Epon resin, Momentive Specialty Chemicals Epoxy Resins (Houston, TX) USA. Semithin sections of 1µm were stained with 1% toluidine blue in borax and examined under a light microscope. Ultrathin sections of 70 nm were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate. Ultrathin sections were examined using SEO (Sumy Electron Optics) model PEM-100 at different magnification. The photos were taken at 75 KV at Electron Microscope Department, Military Medical Academy, Cairo.

- Images were captured by CCD camera JENOPTIK model ProgRes MFcool, CCD camera with 1099 x 694 pixel format as side mount configuration. This camera uses a 1394 fire wire for acquisition. The Image Software capture and Analysis is VideoTest- version 5.0.123.7725 (11&10).

**Scanning electron microscopy (SEM):**
For scanning electron microscopy (SEM), the specimens were washed in phosphate buffer saline (PBS), fixed at room temperature in an aldehyde mixture made up of 4% formaldehyde, 1.25% glutaraldehyde and 10 nmol/l CaCl2 in 0.05 mol/l cacodylate buffer. The samples were dehydrated in ethanol and critical point-dried in a Balzer’s apparatus using carbon dioxide as the transitional fluid. The preparations were mounted on metal stubs with conductive carbon paste. The specimens were coated with Au/Pt under vacuum and examined in a [JEOL (Japan) JSM 6510 lv] scanning electron microscope, Faculty of Agriculture, Al Mansoura University, Egypt. The instrumental conditions for the observations were 15 kW, 0.2 nA, 30 s/frame (photography) (12).

**II. Biochemical study:**
As markers for liver injury. After BDL, blood was collected from rats' tails of all groups with gel & clot activator tube. The blood samples were centrifuged and the supernatant (plasma) was frozen on dry ice and later transferred to 80°C freezer. We estimated the plasma levels of alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin (total & direct &indirect) by use of a spectrophotometer (Roche 917 R autoanalyzer) and commercial kits. Concentration of the biochemical constituents was calculated according to the manufacture instruction (13).
Histo-morphometrical analysis:

The image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Image Analyzing Unit of the Pathology Department, Faculty of Dentistry, Cairo University (Egypt), was used to evaluate optical density for PAS-alcian blue and optical density for chromogranin-A immune reaction. It was measured using the interactive measure menu. Measuring frame of a standard area equal to 118 476.6 mm² was chosen so that PAS-alcian blue and the brown positive immune reaction for chromogranin-A could be seen and masked by blue binary colour to be measured. Ten readings from five non-overlapping sections from each rat of all groups were examined.

IV. Statistical analysis:

The obtained data from biochemical (plasma level of ALT, ALP and bilirubin (total&direct&indirect)) and morphometrical (thickness of mucus) analysis were expressed as mean ± SD (standard deviation) and subjected to one-way analysis of variance (ANOVA) and post hoc test using Statistical Package for the Social Sciences (SPSS) version 16. ANOVA was used for comparison between different groups (more than two groups), with p value less than 0.05 (the level of significance). Least significant difference (LSD) was used to find the statistical difference between the groups when ANOVA was statistically significant (P value <0.05).

RESULTS

Light Microscopic Results:

a. Histology of the liver after BDL:

H&E stained sections of control liver showed the normal architecture of it. Hepatocytes arranged in tightly packed cords radiating from central vein. These hepatocytes had rounded vesicular nuclei and acidophilic cytoplasm. (Fig.1). After BDL, the liver lost its architecture with loss of cords organization. The central veins dilated with detachment of their endothelial lining. Many hepatocytes had vacuolated cytoplasm and some cells had darkly-stained nuclei (Fig.II)

b. Histology of fundic mucosa after BDL:

Histological examination of subgroups 1a, 1b and 1c showed similar histological results. H&E stained sections of control albino rats' fundic mucosa showed the fundic mucosal layers; epithelium, lamina propria containing fundic glands and muscularis mucosa. The glands appeared long straight tubular and perpendicular to the surface epithelium occupying the whole thickness of the lamina propria (Fig. 1). Cholestatic-induced group revealed mucosal discontinuity of the luminal surfaces with exfoliated epithelial cells within the lumena and mononuclear cellular infiltration were seen in the lamina propria (Fig.2). BS supplemented group revealed straight parallel fundic glands with slightly dilated gastric pits (Fig.3). Control group showed fundic mucosa was lined by mucous surface cells with basal oval nuclei that continue into gastric pits. Parietal cells had eosinophilic cytoplasm with central rounded nuclei (Fig.4). While cholestatic induced group showed dilated fundic glands and wide gastric pits. Some of the dilated glands were atrophied and lined by flattened cells with flattened nuclei. Parietal cells had deeply stained shrunken nuclei surrounded by pale vacuolated cytoplasm (Fig. 5). BS supplemented group revealed gastric pits were lined by simple columnar cells with basal oval nuclei (Fig. 6). Control group also showed chief cells had basal rounded nuclei with prominent nucleoli (Fig. 7). Cholestatic-induced group revealed chief cells had deeply stained nuclei and vacuolated cytoplasm. Congested blood vessels were observed (Fig. 8). BS supplemented group showed many chief cells had flattened basal nuclei and vacuolated cytoplasm. Others showed basal rounded nuclei (Fig. 9).

PAS & Alcian blue stained-sections of the control group revealed a strong PAS positive reaction in mucous cells near the lumen and extended down into the gastric pits.
Neutral mucus was bright magenta in color. Also, neck region contained Alcian blue positive reaction for acidic mucus. Acidic mucus in the neck and basal part was stained blue (Fig. 10). Cholestatic-induced group showed weak PAS positive reaction in the surface of mucous cells and gastric pits. Other areas showed strong PAS positive reaction. Neutral mucus was bright magenta. Acid mucus stained by Alcian blue was evident in gastric pits (Fig. 11). BS supplemented group showed strong PAS positive reaction in the lumen that was extending down into the gastric pits. Neutral mucus was deep magenta. Also, neck region contained acid mucus stained by Alcian blue (Fig. 12).

Immunohistochemical stained sections of the control group revealed strong chromogranin A immune reaction that appeared as dark brown color in the cytoplasm of enteroendocrine cells (Fig. 13). Cholestatic-induced group showed noticeable decrease in the intensity of chromogranin A immune reaction that can be observed as a faint brown color in the cytoplasm of some enteroendocrine cells (Fig. 14). BS supplemented group showed strong chromogranin A immune reaction that appeared as dark brown color in the cytoplasm of enteroendocrine cells (Fig. 15).

**Electron microscope examination:**

Ultrathin sections of the control group showed mucous cells had flattened basal euchromatic nuclei and peripheral rim of heterochromatin. Their cytoplasm contained numerous apical electron lucent secretory granules and short cisternae of rER (Fig. 16). Parietal cells had basal rounded euchromatic nuclei. The cytoplasm contained many mitochondria and intracellular canaliculi with their microvilli (Fig. 17). Chief cells had central euchromatic nuclei with peripheral rim of heterochromatin. Their cytoplasm contained basal closely packed cisternae of rER and multiple apical secretory granules of moderate denisty (Fig. 18). Enteroendocrine cells appeared with euchromatic rounded nuclei. The cytoplasm contained numerous granules with variable sizes and electron densities (Fig. 19).

Ultrathin sections of cholestatic-induced group showed many mucous cells with apical aggregation of electron dense mucous granules (Fig. 20). Some parietal cells had dense heterochromatic nuclei. Their cytoplasm contained dilated intracellular canaliculi with prominent microvilli inside them (Fig. 21). Chief cells had ovoid nuclei with much peripheral heterochromatin and dilated cisternae of rER (Fig. 22). Cytoplasm of enteroendocrine cells showed many cytoplasmic vacuoles and few secretory granules. These granules had electron dense core surrounded by electron lucent zone (Fig. 23).

Ultrathin sections of BS supplemented group showed mucous cells which had irregular basal euchromatic nuclei with peripheral heterochromatin. Their cytoplasm was occupied by many electron dense granules (Fig. 24). Parietal cells had ovoid euchromatic nuclei and peripheral thin rim of heterochromatin. Their cytoplasm contained multiple mitochondria and tubulovesicular systems (Fig. 25). Chief cells had basal euchromatic nuclei with peripheral heterochromatin. Their cytoplasm contained electron dense granules and many basal cisternae of rER. Some of them were slightly dilated (Fig. 26). Enterendocrine cells revealed large indented euchromatic nuclei. Their cytoplasm contained multiple small electron dense secretory granules and cytoplasmic vacuoles (Fig. 27).

Scanning of the control group showed many gastric pits. These pits were surrounded by dome-shaped mucous surface cells with well demarcated cell boundaries. Mucus appeared as small globular masses over the surface of cells or as white columns migrating from gastric pits (Fig. 28). Cholestatic-induced group showed loss of demarcation between surface epithelial cells with shedding of mucous cells. The opening of gastric pits showed a honeycomb appearance (Fig. 29). While BS supplemented group showed that the gastric pits were lined and surrounded by dome shaped surface mucous cells with well demarcated cell boundaries.
boundaries. Loss of demarcations between cells was seen at certain areas. Mucus appeared as small globular masses over cells or as a white column migrating from gastric pits (Fig. 30).

Figures I & II are H & E stained sections of liver (x400): Fig. I: Showing tightly packed cords of hepatocytes radiating from central vein (CV). The hepatocytes have rounded vesicular nuclei and acidophilic cytoplasm (arrows) are seen. (Control group) Fig. II: Showing loss of architecture. Dilated central vein (CV) with detachment of the endothelial lining is seen. Many cells have vacuolated cytoplasm (arrow heads) and some cells have darkly-stained nuclei (curved arrows). (Cholestatic-induced group)

Figures 1 to 3 are H & E stained sections (x200): Fig. 1: Showing its three distinctive components: epithelium lining the lumen (arrow heads), underlying connective tissue; lamina
propria containing numerous, narrow and straight fundic glands (arrows) and muscularis mucosa (mm). **Fig. 2:** showing mucosal discontinuity (arrows) of the luminal surface with exfoliated epithelial cells (arrow heads) within the lumen. Mononuclear inflammatory cells (curved arrow) are observed in the lamina propria. **Fig. 3:** showing straight parallel fundic glands (arrows) with slightly dilated gastric pits (P).

**Figures 4 to 9 are H & E stained sections (x400):** **Fig. 4:** showing mucous surface cells (arrows) with basal oval nuclei extending into gastric pits (P). Parietal cells (arrow heads) have eosinophilic cytoplasm with central rounded nuclei. **Fig. 5:** showing dilated fundic glands (d) and wide gastric pits (p). Some of the dilated glands are atrophied and lined by flattened cells with flattened nuclei (arrow heads). Parietal cells (arrows) have deeply stained shrunken nuclei surrounded by pale vacuolated cytoplasm. **Fig. 6:** showing gastric pits (P) are lined by simple columnar cells with basal oval nuclei (arrows). **Fig. 7:** showing chief cells (arrows) have basal rounded nuclei with prominent nucleoli. **Fig. 8:** showing chief cells (arrows) have deeply stained nuclei and vacuolated cytoplasm. Notice, congested blood vessels (v) in the lamina propria. **Fig. 9:** showing many chief cells had flattened basal nuclei and vacuolated cytoplasm (arrows). Others showing basal rounded nuclei (curved arrows).
Figures 10 to 15 are PAS & Alcian blue and chromogranin A immunoreactivity stained sections (x400):  
**Fig. 10:** showing strong PAS positive reaction (arrows) in mucous cells near the lumen extending down into the gastric pits. Neutral mucus is bright magenta in color. Also, neck region contains Alcian blue positive reaction (arrow heads) for acidic mucus. Acidic mucus in the neck and basal part is stained blue.  
**Fig. 11:** showing weak PAS positive reaction (arrow heads) in the surface of mucous cells and gastric pits. Other areas show strong PAS positive reaction (arrows). Neutral mucus is bright magenta. Acid mucus (curved arrows) stained by Alcian blue is evident in gastric pits.  
**Fig. 12:** showing strong PAS positive reaction (arrow heads) in the lumen extending down into the gastric pits. Neutral mucus is deep magenta. Also, neck region contains Alcian blue positive reaction (arrows).  
**Fig. 13:** showing strong chromogranin A immune reaction (arrows) that appear as dark brown color in the cytoplasm of enteroendocrine cells.  
**Fig. 14:** showing noticeable decrease in the intensity of chromogranin A immune reaction that can be observed as a faint brown color (arrows) in the cytoplasm of enteroendocrine cells.  
**Fig. 15:** showing strong chromogranin A immune reaction (arrows) that appear as dark brown color in the cytoplasm of enteroendocrine cells.
Figures 16 to 19 are ultrathin sections: Fig. 16: showing a mucous cell with flattened basal euchromatic nucleus (N) with peripheral rim of heterochromatin. The cytoplasm has numerous apical electron lucent secretory granules (g) and short cisternae of rER (arrows). Fig. 17: showing the parietal cell has basal rounded euchromatic nucleus (N). The cytoplasm contains many mitochondria (m) and intracellular canaliculi (c) with their microvilli (arrows). Fig. 18: showing that chief cell has central euchromatic nucleus (N) with peripheral rim of heterochromatin. Its cytoplasm contains basal closely packed cisternae of rER (arrows) and multiple apical secretory granules of moderate denisty (g). Apical part of parietal cell has tubulo vesicular system (v), intracellular canaliculi (curved arrow) with multiple microvilli (arrow head) and mitochondria (m). Fig. 19: showing an enteroendocrine cell with euchromatic rounded nucleus (N). The cytoplasm has numerous granules (g) with variable sizes and electron densities.
Figures 20 to 23 are ultrathin sections: Fig. 20: showing mucous cells with apical aggregation of electron dense mucous granules (g). Fig. 21: showing a parietal cell has dense heterochromatic nucleus (N). The cytoplasm contains dilated intracellular canaliculi (C) with prominent microvilli (arrows) inside them. Fig. 22: showing a chief cell with heterochromatic nucleus (N) and dilated cisternae of rER (R). Fig. 23: showing an enteroendocrine cell with many cytoplasmic vacuoles (v) and few secretory granules. These granules (arrow heads) have electron dense core surrounded by electron lucent zone.
Figures 24 to 27 are ultrathin sections: Fig. 24: showing a mucous cell has irregular basal euchromatic nucleus (N) with peripheral heterochromatin. Its cytoplasm contains many electron dense granules (g). Fig. 25: showing a parietal cell with an ovoid euchromatic nucleus (N) and peripheral thin rim of heterochromatin. Its cytoplasm contains multiple mitochondria (m) and tubulovesicular (arrows) system. Fig. 26: showing a chief cell has basal euchromatic nucleus (N) with peripheral heterochromatin. Its cytoplasm contains electron dense granules (g) and many basal cisternae of rER (arrows). Some of them are slightly dilated (arrow heads). Fig. 27: showing an enteroendocrine cell with large indented euchromatic nucleus (N). Its cytoplasm contains multiple small electron dense secretory granules (g) and cytoplasmic vacuoles (v).

Figures 28 to 30 are scanning sections: Fig. 28: showing many gastric pits (p). These pits are surrounded by dome-shaped mucous surface cells (arrows) with well demarcated cell boundaries. Mucus appears as small globular masses (curved arrows) over the surface of cells or as white columns (arrow heads) migrating from gastric pits. Fig. 29: showing loss of demarcation between surface epithelial cells with shedding of mucous cells (arrows). The openings of gastric pits show a honeycomb appearance (asterisks). Fig. 30: showing that the gastric pits (curved arrows) are lined and surrounded by dome shaped surface mucous cells (arrows) with well demarcated cell boundaries. Loss of demarcations between cells is seen at certain areas (arrow heads). Mucus (m) appear as small globular masses over cells or as a white column (asterisk) migrating from gastric pits.

Morphometrical and Statistical Results:
- Statistical analysis of thickness of mucus film was done by one way ANOVA (analysis of variance) test and post hoc test LSD (least significant difference).
- Results revealed:
  - Statistically significant difference between the various groups by One way ANOVA as the p value < 0.001. While, LSD for comparison between groups detected a highly significant decrease in the thickness of mucus film of cholestatic-induced group (II) (P<0.001) in comparison to control group (I). A highly significant increase in mucus film
The thickness of BS supplemented group (III) \((P<0.001)\) in comparison to cholestatic induced group (II). On the other hand, non significant increase was detected in group III in comparison to the control group (Table 1& Histogram 1).

Table (1) Comparison between mean values of the thickness of mucus film among studied groups using one way ANOVA test:

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean±SD</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control)</td>
<td>65.3 ± 12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II (Cholestatic induced)</td>
<td>12.4 ± 3.1</td>
<td>55.7</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Group III (BS supplemented)</td>
<td>55.2 ± 11.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD: Standard Deviation 
**: highly significant \((p<0.001)\)

Biochemical and Statistical analysis:
- The levels of liver functions as bilirubin (total, direct and indirect) and liver enzymes as ALT and ALP were estimated. The data obtained were statistically analyzed by one way ANOVA and post hoc test (LSD).
- Results revealed:
  - Statistically significant difference between the various groups by One way ANOVA as the \(p\) value < 0.001. While, LSD for comparison between groups detected a highly significant increase in levels of bilirubin and liver enzymes in cholestatic-induced group (II) \((P<0.001)\) in comparison to control group (I) and a highly significant increase in levels of liver functions in BS supplemented group (III) \((P<0.001)\) in comparison to cholestatic induced group (II) (Table 1&2 and Histogram1&2).
Table (3): Comparisons between mean values of bilirubin of different studied group using ANOVA (analysis of variance) test:

<table>
<thead>
<tr>
<th>Bilirubin</th>
<th>Groups</th>
<th>Control</th>
<th>Cholestatic induced</th>
<th>BS supplemented</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. Bilirubin:</td>
<td>Mean ± SD</td>
<td>0.07 ± 0.01</td>
<td>6.65 ± 2.12</td>
<td>0.16 ± 0.01</td>
<td>47.5</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>D. bilirubin:</td>
<td>Mean ± SD</td>
<td>0.06 ± 0.01</td>
<td>5.9 ± 2.25</td>
<td>0.08 ± 0.01</td>
<td>33.44</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Indirect</td>
<td>Bilirubin:</td>
<td>0.06 ± 0.01</td>
<td>0.87 ± 0.14</td>
<td>0.07 ± 0.01</td>
<td>12.75</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
<td>0.06 – 0.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD: Standard Deviation. **: highly significant (p<0.001)

Histogram (3): Comparison of mean values (±SD) of bilirubin (total&direct&indirect) between studied groups.

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Table (4): Comparisons between mean values of liver enzymes of the different studied group using ANOVA (analysis of variance) test:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control group</th>
<th>Cholestatic induced group</th>
<th>BS supplemented group</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT: Mean ± SD</td>
<td>42.4 ± 0.89</td>
<td>105.5 ± 5.5</td>
<td>41.5 ± 2.5</td>
<td>541.45</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

DISCUSSION

Chronic cholestatic liver diseases are characterized by defective bile acid transport from the liver to intestine caused by primary damage to the biliary epithelium. It may result either from a functional defect in bile formation at the level of the hepatocytes (metabolic cholestasis) or from an impairment in bile secretion and flow at the level of bile ductules or ducts (obstructive type) (14).

There are two methods used for inducing experimental obstructive cholestasis; carbon tetrachloride (CCl4) administration or bile duct ligation (BDL). CCl4 is considered as an extremely toxic method, while, BDL is a safer method comparing to it (8).
In the present study, examination of cholestatic induced group (II) revealed degenerative changes in the fundic mucosa in the form of loss of surface epithelial continuity, exfoliated epithelial cells, dilated fundic glands, widening of gastric pits and some atrophied glands. These results were confirmed by SEM which revealed loss of demarcation between surface epithelial cells with shedding of mucous cells. The opening of gastric pits showed a honeycomb appearance. These results were in consistent with (15). They reported severe gastric mucosal damage in obstructive jaundice cases that is vulnerable to water immersion stress and gastroinvasive agents (eg. aspirin). The gastric mucosal damage was referred to decreased activities of antioxidant enzymes and increase in production of nitric oxide (NO) after bile duct ligation. Accumulated bile acids in these models lead to depletion of reduced GSH and increase in lipid peroxides. Also, retained bile salts induce toxicity and damage of plasma membrane of hepatocytes with depletion of hepatic reduced GSH. Extensive and further release of ROS occur with lack of their scavenger leading finally to gastric mucosal damage(16).

It was found that the gastric mucosa is always exposed to both endogenous and exogenous noxious agents. The ability of the mucosa to resist such agents is based on a dynamic process that involves epithelial secretion, barrier function, mucosal microcirculation and the immune system (17). They reported that NO play a critical role in the pathogenesis of a number of disease processes. Some authors(18) emphasized that there is sequential changes in gastric mucosal NOS activity following BDL with increase in hepatic and systemic NOS. The increase of systemic NO production is probably responsible for the hyperdynamic circulation found in these animals. NO overproduction in such rats was induced either by the endothelial NOS (eNOS), by the inducible NOS (iNOS) enzyme or both enzymes in a sequential manner.

In addition to the role of NO, it was mentioned that prostaglandins (PGs) are involved in the control of gastric microcirculation especially in rats with portal hypertension. The effects of PGs on the rat’s microcirculation are probably independent from those of NO. Pretreatment with L-NAME (NOS inhibitor) resulted in an increase in PGE2 generation that was associated with minor attenuation of the rats’ gastric mucosa damage(19).

On the other hand, dysfunction of the sympathetic nervous system was observed in obstructive jaundice and in hepatic dysfunction. The gastric mucosal circulatory disturbances in obstructive jaundice are attributed to the result of sympathetic nervous system hypofunction due to decreased noradrenaline content. Noradrenaline reduction in the gastric mucosa was inhibited by exogenous PGE2, which was indicated as one of the main factors of ulcer formation in obstructive jaundice(20).

In the same group, submucosal dilated congested blood vessels were explained by (21) who assessed the role of endothelin (vascular endothelial cell derived peptide) as a pro-ulcerogenic mediator. They reported that increased endothelin expression renders the mucosa more vulnerable to damage induced by hydrochloric acid at a concentration tolerated by normal gastric mucosa. Others(18) postulated that advanced lipid peroxidation end-products play a role in increasing endothelin expression in vascular endothelium. They added that endothelin produces marked increase in gastric vascular perfusion pressure, which reflects considerable vasoconstriction. Therefore, reactive vascular congestion is a feature characterizing gastric ulceration.

In the present study, the fundic mucosa of cholestatic- induced group showed mucous cells with apical mucous granules. Scanning of this mucosal surface revealed complete shedding of the surface mucous cells leaving the opening of gastric pits with the shape of a honeycomb appearance. Some researchers (22) stated that cellular hypoxia causes release of lysosomal enzymes into the cells and surrounding extracellular spaces, thereby causing...
damage. Increased mucosal levels and activity of free lysosomal enzymes have been implicated in the pathogenesis of mucosal ulcerations such as induced by sepsis and stress. Also, they found significant increase in the levels of the two lysosomal enzymes acid phosphatase and b-glucuronidase in rats with BDL.

It was presumed that endotoxin (potentially from intestine) not only induces abnormal hemodynamics, but also directly triggers the gastric mucosal damage (23). They revealed imbalance between the protective and damage factors underlies the gastric mucosal injury during portal hypertensive gastropathy (PHG). The results showed that the endotoxin in the plasma from PHG rats significantly increased, which was consistent with the pathological damage in the gastric mucosa.

The same group also revealed parietal and chief cells affection in both light and TEM. Parietal cells had shrunken deeply stained nuclei surrounded by wide intracellular canaliculi in vacuolated cytoplasm. While chief cells had vacuolated cytoplasm with condensed heterochromatic nuclei and dilated cisternae of rough endoplasmic reticulum. It was reported that decrease in chief cell and parietal cell mass and acid secretion in congestive gastropathy(24). They attributed this decrease to diminution of the gastric mucosal protective factors which induced a decrease in acid and pepsin secretion resulting in gastric mucosal damage. On the other hand, (25) confirmed the potent inhibitory effect of cholestasis and cirrhosis on acid and pepsin secretions. They referred this effect to increase the levels of gastric tissue NO metabolites. While, the role of gastric secretion in cirrhosis is controversial; some studies reported reduced acid secretion while others reported normal production. Some reports (26) revealed decrease in gastric acid output in rats with long-term BDL and they referred that to either defective function of the parietal cells or diminution of parietal cell number.

In the present work, cholestatic-induced group showed congested blood vessels and cellular infiltration in the lamina propria after BDL. Similar results were explained by oxidative stress that involved in the pathogenesis of many gastric organic disorders such as gastritis, gastric ulcers and gastric cancer(27).

The key event in the pathophysiology of obstructive jaundice-associated complications is endotoxaemia of gut origin because of intestinal barrier failure. The excessive presence of endotoxins in the portal and systemic circulation stimulates a systemic inflammatory response characterized by the release of cytokines and other proinflammatory cytokines such as TNF-a, , IL-1, IL-6, interferon–gamma (INF–c), NO and oxygen free radicals(28).

Some researchers (26) correlated between the gastric mucosal damage and neutrophilic infiltration. Activated neutrophils produce many enzymes and free radicals that induce further mucosal damage and are considered as an aggressive factor in ulcer formation. Also, they recorded defects in neutrophil functions such as impairment of adhesion, defective phagocytosis, and increase in superoxide generation and chemotaxis with increased their number.

The lamina propria of the same group (II) also showed eosinophilic infiltration with its characteristic electron dense core granules. (29) explained the role of eosinophil peroxidase in oxidative overload implicated in pathogenesis of gastric ulceration. This enzyme belongs to halo-peroxidases that act on bromide ions, generating hypobromous acid. Eosinophil peroxidase is also capable of oxidizing thiocyanate (SCN–). These oxidative compounds play a role in gastric ulcer pathology. On the opposite side, (30) reported that the cytotoxic molecule; eosinophil cationic protein (ECP) which is secreted by the activated eosinophils and may contribute to inflammatory changes seen in chronic gastritis. Whereas there is no proof that they play a role in ulcer development.
In the current study, PAS-alcian blue stained sections of the cholestatic- induced group revealed negative reaction in the surface epithelium and the mucous neck cells in the neck region of the fundic glands. TEM revealed mucous cells with apical electron dense granules. Also, SEM showed loss of the normal mucous sheet covering the surface mucous cells with some mucous patches. Globular mucous form disappeared. These findings were in agreement with (31), who attributed these changes to occurrence of damage in the gastric mucosal barriers. The first line of defense in the stomach mucus was decreased due to suppressed prostaglandin production and damage of the surface epithelial cells and mucus neck cells. It was reported that this protective barrier is impaired in patients with cirrhosis. On the other hand, decreased mucus secretion allows hydrogen ions and pepsin to diffuse into the mucosa from the lumen. Back diffusion of acid and pepsin into the tissues stimulates further acid and pepsin secretion, decreases mucosal blood flow and decreases gastric motility. The acid also damages connective tissue and submucosal capillaries to cause focal mucosal hemorrhage (32).

Biochemical results of the present study showed high significant increase in liver functions in cholestatic- induced group in comparison to control and BS supplemented group. It was found that certain ATP-binding cassette (ABC) transporter proteins as multidrug resistance protein (Mrp3) expression is increased after common bile duct ligation. This could provide basolateral efflux of organic anions like bilirubin explaining the appearance of conjugated pigment in plasma and urine in obstructive cholestasis. Therefore, the reciprocal regulation of Mrp2/Mrp3 provides an alternative mechanism for the excretion of toxic bile salts and other Mrp2 substrates during cholestasis (33).

In addition, (34) reported that patients who have secondary biliary cirrhosis exhibit hyperbilirubinemia due to biliary obstruction resulting from liver damage with higher ranges for aminotransferases. High levels of these enzymes are largely caused by the necrosis of tissues that are rich in aminotransferases.

It was recorded that serum total bilirubin level dramatically elevated until it reached its peak after 4 weeks of bile duct ligation (35). Severe inflammation in both blood and hepatic tissue is determined by drastic increases of hepatocyte aminotransferases (ALT peaked on week 4, while AST peaked on week 1 of BDL). The decrements of serum total bilirubin and hepatic aminotransferases following their peaks are in consistence with previous studies. The release of large amounts of liver enzymes in the bloodstream is associated with a loss of integrity with consequent tissue apoptosis and the necrosis of hepatocytes. Elevated serum levels of these enzymes mean cellular damage, (34).

In the current study, BS supplemented group showed apparently normal fundic mucosa. Numerous researches have been done to identify plant-derived natural substances and understand the mechanisms of their pharmacological actions. BS extract increases the activity of antioxidant enzymes (catalase, glutathione peroxidase, and glutathione-s-transferase) and acts as a free radical scavenger. It acts as anticancer because it can modulate the activities of molecular targets including p53, p73, PTEN, STAT3, PPAR-g, activation of caspases and generation of ROS have been demonstrated. It also suppresses inflammatory mediators, leukotrienes, prostaglandins, and B cell-mediated immune response while balances Th1/ Th2 responses and potentiates T cell and natural killer cell-mediated immune responses (36).

Moreover, these results were in accordance with (37). They recorded the protective effects of BS on cholestatic liver injury in rats. Their results also suggested that the reduction of neutrophil infiltration and oxidative stress in the liver was probably responsible for this protective effect. Additionally, it was proved that Thymoquinone (TQ), the active principle of BS oil, has protective effect on sodium fluoride-induced hepatotoxicity. TQ improved the
oxidative stress by increase the antioxidant status and reduced the alterations in biochemical parameters. (38)

Other authors attributed this protective effect to the ability of TQ to antagonize increased lipid peroxidation (LPO) and in turn stabilizing the integrity of the cellular membranes and decreasing the leakage of liver enzymes. Also, it was shown that TQ (50 mg/kg body weight) significantly inhibited tamoxifen-induced hepatic glutathione depletion and normalized the activity of SOD (39).

In another study, the effects of TQ either alone or coadministrated with omeprazole on gastric mucosal ischemia/reperfusion injury were investigated in rats. The results revealed that TQ had gastroprotective effects which were mediated by inhibiting proton pump, acid secretion and neutrophil infiltration, and increasing mucin secretion, and nitric oxide production (40).

In conclusion, the current study revealed that cholestasis induction led to several histological and immuno-histochemical alterations in the gastric fundic mucosa of male albino rats. Also, BS as a treatment improved these histological and immunohistochemical changes of gastric fundic mucosa.

In addition, we need further studies upon the possible protective effect of antioxidants and trace elements as vitamin E, ascorbic acid, zinc and copper that could be used to inhibit oxidative stress elicited by cholestasis.

References: