Histological study of the protective role of ginger on piroxicam-induced liver toxicity in mice

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Abstract
Background: Piroxicam is a non-steroidal anti-inflammatory drug widely used in rheumatic diseases. It has analgesic and anti-inflammatory activity, and is one of the drugs being introduced in clinical practice. Piroxicam-hepatotoxicity has been reported as one of its principal side effects. Several natural antioxidants were found to be effective against drug induced toxicity. Ginger is known by its antioxidant activities and hepatoprotective properties. The present study aimed at studying the protective effect of Ginger on Piroxicam-induced histopathological changes in livers of male mice.

Methods: Forty adult mice were randomly divided into 4 groups: Group I served as the control group. Group II received Ginger orally in a dose of 200 mg/kg per day for four weeks. Group III received Piroxicam intraperitoneally in a dose of 0.3 mg/kg per day for four weeks. Group IV received (Piroxicam + Ginger). At the end of the experiment, liver functions were estimated and then the liver was removed, and sampled for histopathological, immunohistochemistry and biochemical studies.

Results: Administration of ginger decreased elevated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) and immunoexpression of the proapoptotic protein (Bax), induced by piroxicam. It increased immunoexpression of the antiapoptotic protein (Bcl2). It also ameliorated the morphological changes induced by piroxicam.

Conclusion: Piroxicam has toxic effects on the liver as indicated by biochemical, histological and immunohistochemical results. Ginger has protective effects against piroxicam-hepatotoxicity by reducing serum marker enzymes, liver fibrosis and apoptosis.

Keywords: Antioxidants; Ginger; Liver damage; Piroxicam

1. INTRODUCTION

The susceptibility of the body to the oxidative damage is determined by balance between the production and scavenging of reactive oxygen species (ROS) or free radicals. The antioxidant defense mechanisms of an organism can minimize the production of free radicals, and protect against the oxidative damage, but it may not be enough to totally prevent the damage. Many drugs can generate free radicals thus inducing oxidative stress. These free radicals may cause damage to tissue proteins, nucleic acids, and membrane lipids. The level of defense mechanisms may not increase after drug intake, therefore scavenging of free radicals is insufficient and tissue injury is produced.1

Rheumatic diseases are worldwide. They are considered to cause many social and medical problems. Prevention and treatment of rheumatic diseases is one of the most important challenges concerned with public health problems. A variety of non-steroidal anti-inflammatory drugs (NSAIDs), have been clinically used as antirheumatics. NSAIDs represent one of many chemical agents having useful analgesic and antiinflammatory characteristics.2

Piroxicam is a common NSAIDs used for treatment of inflammatory and rheumatic disorders. It has analgesic and antipyretic activity.3

The role of liver in drug metabolism makes it susceptible to toxic injury. As piroxicam is metabolized in liver, there is possibility of hepatic injury. The toxicity produced by piroxicam is mediated through oxidative stress, which leads to lipid peroxidation (LPO) and free radical generation.4 Recently, Knottenbelt et al.5 found that a significant inhibition of proliferation and induction of apoptosis occur when piroxicam is used in concentrations exceeding maximum recommended doses.6

Several natural antioxidants were found to be effective against various human ailments. Their medicinal uses have been increasing in developed countries. They were used to protect against drug toxicity to strengthen endogenous antioxidants defenses and restore optimal balance by neutralizing reactive oxygen species.7

Ginger belonged to Zingiberaceae family, is a free radical scavenger.8 Several studies have demonstrated that ginger possesses numerous therapeutic properties. It has analgesic and anti-inflammatory properties.9 The hepatoprotective role of ginger against toxicity caused by CCl4 and acetaminophen was proved as it improved elevated serum transaminases in rat study.10

Hence, this work was performed to evaluate possible protective effects of ginger on piroxicam-induced histopathological changes in livers of male mice.

2. METHODS

2.1. Animals

The present study was carried out on 40 healthy adult male
mice weighing from 30 to 40 g and were purchased from the animal house of Assiut Faculty of Medicine, Assiut University, Egypt. The mice were housed in polypropylene cages under standard lightening in a temperature-controlled room (25 ± 2 °C) and had free access to laboratory food and water throughout the experiment. They were acclimated to their environment for at least two weeks before starting the experiment. All animal procedures were approved by the local Institutional Animal Ethical Committee of Faculty of Medicine, NBU, KSA.

2.2. Experimental design

After acclimatization period, mice were randomly divided into four groups (ten mice in each) as follows:

- **Group I (Control group)**: Received daily injection of normal saline (vehicle) intraperitoneally (i.p.) for 4 weeks.
- **Group II**: Received Ginger powder (Sigma–Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 200 mg/kg/day dissolved in normal saline by gastric gavages for 4 weeks.11
- **Group III**: Received piroxicam powder (Sigma–Aldrich Chemical Co. St. Louis, MO, USA) at a dose of 0.3 mg/kg/day dissolved in normal saline injected intraperitoneally for 4 weeks.12
- **Group IV**: Received ginger (200 mg/kg/day) and piroxicam injection (0.3 mg/kg/day) for 4 weeks.

Twenty-four hours after last drug regimen, mice were anesthetized with intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight). The chest wall was incised to explore the heart. 5 ml of intracardiac blood was drawn and serum was separated for estimation of serum marker enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)].

Animals were sacrificed by decapitation; an abdominal incision was made to remove the liver. This was perfused with a fixative solution (2% paraformaldehyde and 2% glutaraldehyde solution) in 0.1 M phosphate buffer pH 7.2 and then sampled for histopathological studies.

2.3. Assessment of hepatotoxicity

Levels of AST, ALT, and ALP were measured using standard laboratory techniques to assess hepatotoxicity using commercial kits in an Olympus AU400 Chemistry Analyzer (Olympus Corp., Tokyo, Japan). Results are expressed as units/uL.

2.4. Histological and immunohistochemical examination

Liver from each animal was kept in 10% of neutral buffered formalin for 24 h. It was then processed, embedded in paraffin wax and sections of 4 mm thickness were taken using a microtome. These sections were stained with the following stains and examined under light microscope:

- A) Hematoxylin and eosin stain (H&E): for the general histological study.
- B) Periodic acid Schiff’s stain (PAS) for demonstration of liver glycogen.
- C) Masson’s trichrome stain for demonstration of collagen fibers.

Liver sections were immunohistochemically stained to assess immunoeexpression of proapoptotic protein (Bax), and antiapoptotic protein (Bcl2). Paraffin sections of liver were cut at 4 μm thickness on positively charged slides. Sections were incubated with a monoclonal antibody against Bax and Bcl2 (Dako, Carpinteria California, USA); in a dilution of 1:200. Cells displaying brown precipitation were considered positive for Bax and Bcl2 expressions.

2.5. Quantitative morphometric measurement

Leica Qwin 500 C Image analyzer computer system (Leica Imaging System LTD., Cambridge, England) in Central Research Lab, Assiut Faculty of Medicine, Egypt was used to obtain morphometric data in this study. Ten non-overlapping fields in slides of each animal in each group were examined. Measurements were performed on 6 mm thick H&E stained sections to estimate:

1. Diameter of blood sinusoids in control and treated groups (At magnification 400).
2. Diameter of central veins in control and treated groups (At magnification 400).

2.6. Statistical analysis

All analyses were performed using the software Statistical Package for Social Sciences version 17 (SPSS Inc, Chicago, IL, USA). Data was presented as mean ± standard deviation (SD). Comparisons between two groups were analyzed by unpaired Student “t” test. Probability of chance (p value) < 0.05 was considered statistically significant.

3. RESULTS

None of experimental mice died during the experiment period (4 weeks).

3.1. Biochemical results

There was significant increase in serum ALT, AST and ALP in piroxicam-treated mice. Administration of ginger along with piroxicam showed significantly lower enzyme levels compared to piroxicam-treated group. Mice treated with ginger alone showed normal values of enzymes (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (Ginger)</th>
<th>Group III (Piroxicam)</th>
<th>Group IV (Piroxicam + Ginger)</th>
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<tr>
<td>AST (uL)</td>
<td>54.8 ± 0.77</td>
<td>53.6 ± 0.52</td>
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<td>ALT (uL)</td>
<td>17.8 ± 0.61</td>
<td>17.5 ± 0.61</td>
<td>25.5 ± 0.78</td>
<td>19.8 ± 0.81</td>
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<td>ALP (uL)</td>
<td>73.6 ± 0.68</td>
<td>72.4 ± 0.75</td>
<td>86.6 ± 1.83</td>
<td>74.5 ± 0.88</td>
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Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student’s t test at p < 0.05.

* p < 0.001 compared with the control group (group I).
* p < 0.001 compared with the treated group (group II).

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3.2. Histological results

3.2.1. Hematoxylin and eosin stain
The architecture of hepatic tissue of control, and ginger-treated mice were more or less similar, showing hepatocytes arranged in cords radiating from the central vein. They are polygonal cells with pale vesicular nuclei and prominent nucleoli. Some hepatocytes were binucleated. They have an eosinophilic granular cytoplasm. Blood sinusoids were found as a network between the plates of hepatocytes converging towards the central vein. There were portal areas containing connective tissue stroma and portal triad (Fig. 1A).

In piroxicam group, liver sections revealed dilatation of central veins and blood sinusoids. Many hepatocytes were ballooned with central faint nuclei and vacuolated cytoplasm. Other hepatocytes appeared small with deeply stained acidophilic cytoplasm and dark nuclei (Fig. 1B). Some central veins were surrounded by cellular infiltration (Fig. 1C). Some hepatocytes showed early signs of apoptosis, with fragmented nuclei, hazy vacuolated cytoplasm and indistinct cell boundaries (Fig. 1D). Apoptotic hepatocytes were also detected, showing nuclear and cytoplasmic condensation into deeply stained apoptotic bodies (Fig. 1E).

When ginger was administered with piroxicam, liver sections appeared somewhat normal in histological architecture. Almost normal hepatocyte appearance was observed both in central as well as in peripheral zones of hepatic lobules (Fig. 1F).

3.2.2. Periodic acid schiff’s (PAS) stain
Control and ginger-treated mice showed abundant glycogen content diffusely distributed throughout the hepatic lobules. Glycogen granules appeared as heavy magenta red deposits filling the cytoplasm of hepatocytes (Fig. 2A).

In piroxicam group, liver sections showed evident depletion of glycogen stores of liver. Many hepatocytes showed a cytoplasm either partially or completely devoid of glycogen granules (Fig. 2B).

In combined piroxicam and ginger group, liver sections revealed restoration of PAS staining tissue to normal. The sections showed abundant glycogen content similar to control group (Fig. 2C).

3.2.3. Masson’s trichrome stain
In control and ginger group, liver Sections revealed very thin layer of collagen fibers around central veins (Fig. 3A). Piroxicam-treated mice showed deposition of marked amount of collagen fibers around central veins and blood sinusoids (Fig. 3B). Furthermore, sections showed bundles of collagen fibers dissecting the parenchyma and extending inside the liver lobules (Fig. 3C).

In combined piroxicam and ginger group, liver sections appeared normal (Fig. 3D).

3.3. Immunohistochemical results

3.3.1. Immunostaining of Bax antigen
In control and ginger groups, liver tissues revealed negative immunostaining reaction for Bax (Fig. 4A). Piroxicam-treated mice, revealed dark brown granules in their cytoplasm throughout most cells of liver parenchyma (Fig. 4B). Combined piroxicam and ginger group showed Bax activity more or less similar to the control group (Fig. 4C).

3.3.2. Immunostaining of Bcl2
In control and ginger group, hepatic tissue showed moderate to marked Bcl2 reaction in the cytoplasm of hepatic cells (Fig. 5A). In piroxicam-treated mice, Bcl2 immunostaining was markedly less intense in cytoplasm of hepatic cells (Fig. 5B). Combined piroxicam and ginger group showed increased Bcl2 expression compared with those of piroxicam-treated group (Fig. 5C).

3.4. Quantitative morphometric measurement
Diameters of blood sinusoids and central veins were significantly increased in piroxicam-treated mice. Combined piroxicam and ginger group showed significantly decreased diameter of blood sinusoids and central veins compared to piroxicam-treated group. Mice treated with ginger alone did not show difference when compared to normal values (Table 2).

4. DISCUSSION
Piroxicam is the most famous hepatotoxic drug in the experimental study. One of the main causes of liver toxicity is peroxidative degradation of the lipid membrane. Previous studies have declared that the mechanism of piroxicam hepatotoxicity is due to impairment of mitochondrial ATP synthesis and active metabolites production, mainly 5-hydroxy piroxicam, which causes direct toxicity to the hepatocytes.19

In the current study, the hepatoprotective effect of ginger on piroxicam-induced hepatotoxicity was investigated. All major active constituents of ginger have anti-inflammatory and antioxidant action.14 The antioxidant activity of ginger is either due to reduction or prevention of formation of free radicals. In addition, ginger oil has a protective effect against DNA damage and could act as oxygen radical scavenger. Ginger was proved as an efficient herbal medicine with minimal side effects.15

Marked elevation of the levels of liver enzymes (AST, ALT, and ALP) indicated significant liver damage in piroxicam-induced hepatotoxicity. The liver damage may be due to free radical production by the drug. These results are consistent with the study of Pandit et al.16 This study revealed liver enzymes elevation in patients taking regular diclofenac regimen. Hussaini and Farrington17 documented the relative rise of ALT and ALP accompanying the liver injury.

Levels of liver marker enzymes were significantly decreased in combined piroxicam and ginger group as compared with the piroxicam group which suggest its ability to protect the liver from destruction. These data are in agreement with previous studies proving improvement of the liver enzymes (ALT, AST and ALP) following treatment with ginger in CCl4, induced liver toxicity.11 Another study showed significant reduction in serum levels of transaminases in group received combined cisplatin and ginger indicating that cisplatin-induced impairments of liver function is effectively improved by ginger.16

Histological examination of H&E stained sections of piroxicam-treated mice revealed prominent histological alterations in the liver. These changes were in the form of vacuolation of the cytoplasm, necrosis of some hepatocytes, and dilatation of central veins and blood sinusoids. There was a significant difference in histological score between piroxicam group and control group. The results of the present study agreed with the study of Bessone who found liver injury in the form of necrosis after the use of Non-steroidal anti-inflammatory drugs (NSAID).19 Durham et al.20 noticed that collection of injurious substances in the cells leads to vacuolar degeneration. A third study stated
Fig. 1 (Panel A) A photomicrograph of normal architecture of the liver in the control group showing polyhedral shaped hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm radiating from the central vein (CV) and separated by blood sinusoids (s). The portal triad [portal venule (PV), hepatic arteriole (A) and bile ductile (b)] is enclosed in a fine connective tissue stroma within the portal tract. (H&E, scale bars = 40 μm). (Panels B–E) Photomicrographs of liver of piroxicam treated mice. (Panel B) Many of the hepatocytes are ballooned with central faint nuclei and vacuolated cytoplasm (arrows). Other hepatocytes appeared small with deeply stained acidophilic cytoplasm and dark nuclei (arrow heads). Blood sinusoids appear dilated (s). (Panel C) Inflammatory cellular infiltration (arrows) is seen within the liver tissue. (Panel D) Hepatocytes with early signs of apoptosis. These hepatocytes showed fragmented nuclei with hazy vacuolated cytoplasm and indistinct cell boundaries (arrows). (Panel E) Some apoptotic hepatocytes with nuclear and cytoplasmic condensation into deeply stained apoptotic bodies (thin arrows), dilatation in blood sinusoids (S) and vacuolation of cytoplasm of some hepatocytes (thick arrows). (H&E, scale bars = 40 μm (panels B–E)). (Panel F) A photomicrograph of liver of piroxicam + ginger group with normal looking hepatocytes, central vein (CV), blood sinusoids (s) and portal triad. (H&E, scale bars = 40 μm).
that fatty degeneration, vacuolations, and sinusoidal dilations were noticed in liver of mice treated with piroxicam.

Results of the present study also showed lymphocytic cellular infiltration in the liver tissue. This is supported by El-Banhawy et al. who suggested that presence of leucocytes, in general, and lymphocytes, in particular, is an eminent reflex of the body tissues exposed to any injurious substances. McCafferty et al. suggested that increase number of leucocytes and its adherence to the endothelial wall of the blood vessels plays an important role in the pathogenesis of NSAID associated injury.

Marked histological amelioration was observed in liver tissue of mice treated with a combination of piroxicam and ginger compared to piroxicam group as most of the hepatocytes, central veins, blood sinusoids and portal triads appeared normal having the same characteristics of the control group. The current results are in agreement with Valko et al. who proved that ginger extract can control the quantity of free radicals and the peroxidation of lipids. Thus, it could prevent or decrease the damage in the human body caused by free radicals. Masuda et al. denoted that fresh ginger rhizome contains gingerols (polyphenols) identified as the major active component, zingerone, dehydrozingerone, shogaols and some related phenolic ketone derivatives which exhibit potent antioxidant activities.

The present work revealed that treatment with piroxicam caused a marked decrease in the amount of hepatic glycogen, especially at the periphery of the lobule. These results came in agreement with Tasci et al. who found that glycogen depletion started in zone 1 hepatocytes at the periphery of the hepatic lobule. It occurred secondary to the piroxicaminduced lipid peroxidative damage to the liver mitochondria, present in larger amounts in zone 1 hepatocytes. Accordingly, the energy machinery within the cells became endangered resulting in compensatory enhancement of glycogenolysis.

Marked amelioration in the amount of hepatic glycogen was observed in the group treated with combined piroxicam and ginger compared to piroxicam group. This occurs secondary to inhibition of the lipid peroxidation damage to the mitochondria, thus inhibiting glycogenolysis. The potential hepatoprotective role of ginger may be associated with antioxidant constituents such as 6-gingerol, 6-shogoal, 6-paradol, zingerone, and some related phenolic ketone derivatives working individually or in synergy.

The results of the present study showed that piroxicam caused deposition of excessive amount of collagen fibers around the central vein, blood sinusoids and portal tracts and aggregation of collagen fibers inside the liver lobules. This may be explained by activation of hepatic stellate cells that produce fibrotic neomatrix. Exposure to hepatic toxins enhances the fibroblast growth factors-1 and -2 (FGF-1, FGF-2) which in turn could stimulate the hepatic stellate cells directly to enhance the deposition of collagen type I in the extracellular matrix.

Normal deposition of few fine collagen fibers around central vein and blood sinusoids was observed in the group treated with combined piroxicam and ginger. These results were confirmed by Yacout et al. who denoted significant amelioration in the hepatic fibrosis caused by CCl4 on concomitant oral administration of the crude ethanolic ginger extract at a dose of 200 mg/kg body weight, every day for one week. The study declared that the exhibited hepatoprotective activity is due to the presence of antioxidant compounds in ginger extract.

Morphometric studies in the present study revealed significant increase in the diameter of central veins and blood sinusoids in piroxicam-treated group. This may be attributed to the increase in the level of nitric oxide (NO). Nitric oxideinduced vasodilatation was also reported by Hingorani.

In the group treated with combined piroxicam and ginger, Morphometric studies revealed normal diameter of central veins and blood sinusoids. This can be attributed to the antioxidant properties and the potent hepatoprotective effect of ginger.

It could be concluded that piroxicam has toxic effects on the liver as indicated by biochemical, histological and immu-
Ginger has protective effects against piroxicam-induced hepatotoxicity by reducing serum marker enzymes, liver fibrosis and apoptosis.

**ACKNOWLEDGMENTS**

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Fig. 4 (A) Negative immunostaining reaction of Bax in the liver tissue of the control group. (B) Strong positive immunostaining reaction of Bax in the liver tissue of the piroxicam-treated group. (C) Negative immunostaining reaction of Bax in the liver tissue of group treated with piroxicam + ginger (Bax, immunostaining, scale bars = 40 μm).

Fig. 5 (A) Strong positive immunostaining reaction of Bcl2 in the liver tissue of the control group. (B) Weak positive reaction of Bcl2 in the liver tissue of piroxicam-treated group. (C) Strong positive immunostaining of Bcl2 in the liver tissue of group treated with piroxicam + ginger (Bcl2, immunostaining, scale bars = 40 μm).

Table 2

<table>
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<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
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<th>Group III (Piroxicam)</th>
<th>Group IV (Piroxicam + Ginger)</th>
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<tbody>
<tr>
<td>Diameter of BS (μm)</td>
<td>7.2 ± 1.08</td>
<td>7.3 ± 1.09</td>
<td>16.4 ± 2.29</td>
<td>7.6 ± 1.19</td>
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<tr>
<td>Diameter of CV (μm)</td>
<td>97.3 ± 3.04</td>
<td>97.6 ± 3.09</td>
<td>217 ± 4.35</td>
<td>98.9 ± 3.22</td>
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Data is expressed as mean ± standard deviation. Results were statistically analyzed by using Student’s t test at p < 0.05.

1 p < 0.001 compared with the control group (group I).
2 p < 0.001 compared with the treated group (group III).
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