Original Article

Antioxidant Effect of Sepia Ink Extract on Extrahepatic Cholestasis Induced by Bile Duct Ligation in Rats

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Abstract

Objective The aim of our study was to assess the complications of hepatic fibrosis associated with bile duct ligation and the potential curative role of sepia ink extract in hepatic damage induced by bile duct ligation.

Methods *Rattus norv*egicus rats were divided into 3 groups: Sham-operated group, model rats that underwent common bile duct ligation (BDL), and BDL rats treated orally with sepia ink extract (200 mg/kg body weight) for 7, 14, and 28 d after BDL.

Results There was a significant reduction in hepatic enzymes, ALP, GGT, bilirubin levels, and oxidative stress in the BDL group after treatment with sepia ink extract. Collagen deposition reduced after sepia ink extract treatment as compared to BDL groups, suggesting that the liver was repaired. Histopathological examination of liver treated with sepia ink extract showed moderate degeneration in the hepatic architecture and mild degeneration in hepatocytes as compared to BDL groups.

Conclusion Sepia ink extract provides a curative effect and an antioxidant capacity on BDL rats and could ameliorate the complications of liver cholestasis.

Key words: Bile duct ligation; Hepatic fibrosis; Oxidative stress; Liver collagen percentage; Histopathological examination

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INTRODUCTION

The liver is one of the largest organs in human body and the chief site for intense metabolism and excretion. Therefore, it has a surprising role in the maintenance, performance, and regulation of homeostasis of the body. The liver is involved with almost all the biochemical pathways to growth: defense against disease, nutrient supply, energy provision, and reproduction[1]. Chronic liver diseases are major global health problems, causing approximately 800,000 deaths per year worldwide[2].

Bile duct ligation (BDL) is a typical model of biliary disease in animals, where complete biliary obstruction causes cholestatic injury to the liver[3]. Cholestasis is characterized by an abnormal accumulation of bile and a defect in the process of bile acid transport, leading to impairment in bile formation, which results in progressive liver injury culminating in cirrhosis and liver failure[4].

Bile duct ligation (BDL) in rats is characterized by rapidly progressing biliary fibrosis. The initial stages are represented by acute cholestasis, in which oxidative stress and inflammation play essential roles. Generation of reactive oxygen species (ROS) increases, while the antioxidant capacity of the liver tissue diminishes. It seems that oxidative stress may

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not be the primary aggressor, rather the consequence of inflammation and bile acid accumulation[5].

The reduction of oxidative stress and clearing of ROS are major prerequisites of aerobic life[6] and could play an essential role in the treatment of obstructive cholestasis[7]. Natural products have traditionally played an important role in drug discovery and were the basis of most early medicines[8].

Marine organisms present a rich source of biologically active compounds. More than 15,000 marine natural products were reported to have been isolated between 1965 to 2005[9]. These compounds could play an important role in defense mechanisms against biotic and abiotic stress. Sepia ink has proved to be an alternative medicine and has a wide range of therapeutic applications[10]. It has been shown to contain a variety of melanogenic enzymes including tyrosine, a dopachrome-rearranging enzyme[11], and peroxidase. In addition, melanin and dopamine have been detected in squid ink, with dopamine occurring at varying concentrations, ranging from 1 to 500 nmol/L[12]. Sepia ink has anti-oxidant[13], anti-radiation, anti-retroviral, and anti-bacterial properties[14]. Background research has shown that sepia ink not only promotes thromboxane and kills cancer cells, but also elevates the number of leukocytes[13]. In addition, sepia ink contains purified peptidoglycan, which has anticancer properties[15]. Therefore, the aim of our study was to investigate the complications of the cholestatic liver injury induced by bile duct ligation and the potential curative role of sepia ink in hepatic damage caused by this model of liver fibrosis.

METHODS

Experimental Animals

Male albino rats (*Rattus norvegicus*) weighing 150-160 g were used for the study. The animals were purchased from National Research Center (NRC, Giza, Egypt). Rats were housed under standard conditions, and all of the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC, Faculty of Science, Cairo University, Egypt CUFS/F/PHY/04/13), in accordance with international guidelines for care and use of laboratory animals. Rats underwent bile duct ligation according to previously described methods[16]. Laparotomy was performed under antiseptic conditions, and rats were anesthetized with ketamine[17]. In Sham-operated rats, an abdominal incision was made, but ligation was not performed. In BDL rats, the common bile duct was doubly ligated and transected between the ligated sites.

Chemicals and Reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), ketamine, direct red 80, fast green, and Picric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kits for all biochemical parameters were purchased from Bio-diagnostic Company (Giza, Egypt).

Preparation of Cuttlefish Sepia Ink Extract

Fresh cuttlefish (*Sepia officinalis*) were purchased directly from a fishmonger. They were rapidly transferred to the laboratory, where they were dissected, and their ink was collected and diluted immediately with an equal volume of distilled water, and then mixed sufficiently. The admixture was collected immediately, concentrated, and lyophilized to a black residue using a lyophilizer (LABCONCO, shell freeze system, USA).

Determination of Antioxidant Activity (scavenging activity of DPPH radical)

The free radical scavenging activities of each extract and ascorbic acid were analyzed by the DPPH assay[18]. A 1.0 mL of the test extract, at gradient final concentrations of 10-60 mg/mL, was mixed with 2 mL of 0.3 mmol/L DPPH solution in MeOH in a cuvette. The absorbance was taken at 517 nm after 20 min of incubation in the dark at room temperature. The experiment was done in triplicates. The percentage antioxidant activity was calculated as follows:

Antioxidant Activity (AA)%=

$$\left[1 - \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right)\right] \times 100$$

Where: Abs_{sample} was the absorbance of sample solution (2.0 mL) + DPPH solution (1.0 mL, 0.3 mmol/L), Abs_{blank} was the absorbance of Methanol (1.0 mL) + sample solution (2.0 mL), $Abs_{control}$ was the absorbance of DPPH solution (1.0 mL, 0.3 mmol/L) + methanol (2.0 mL).

Toxicity Study (OECD 420)

Acute toxicity was calculated per Organization for Economic Co-operation and Development (OECD)

guideline 420 (Fixed dose method)[19].

Experimental Design

Fifty-four male albino rats were separated into two main groups: the Sham-operated control (18 rats) and bile duct ligated (BDL) group (36 rats).

After 14 days, the rats of the Sham-operated group were divided into 3 subgroups (6 rats/ subgroup), and animals of these three subgroups were administered distilled water orally for 7, 14, and 28 d. After 14 days, the rats of the Bile duct ligated (BDL) group were divided into 6 subgroups (6 rats/subgroup), and animals of the first three subgroups were administered distilled water orally for 7, 14, 28 d. The rats in the other BDL subgroups were treated orally with ink extract (IE) (200 mg/kg body weight) for 7, 14, and 28 d. At the end of each experimental period, rats were euthanized under chloroform vapor and sacrificed after being fasted overnight. A blood sample from each group was collected without anticoagulant in centrifuge tubes. The liver was removed and immediately blotted using filter paper to remove traces of blood and then divided into two parts. The first part was stored at -80 °C for biochemical analysis, while the second part was suspended in 10% formal saline for fixation in preparation of histopathological examination.

Biochemical Analysis

The collected blood samples were centrifuged at 2000 g for 20 min. The collected serum was stored at -20 °C until used for biochemical assays. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT)[20], serum total protein[21], serum albumin[22], alkaline phosphatase activity (ALP)[23], serum total bilirubin[24], and γ-glutamyltransferase (GGT)[25] were determined using Bio-diagnostic assay kits according to the manufacturer's instructions (Giza, Egypt). Globulin and albumin/globulin (A/G) ratio were calculated.

Determination of Oxidative Stress Parameters

Liver tissue was homogenized (10% w/v) in ice-cold 0.1 mol/L Tris-HCl buffers (pH 7.4). The homogenate was centrifuged at 2000 g for 15 min at 40 °C, and the resultant supernatant was used to determine oxidative stress markers. Levels of lipid peroxide[26], reduced glutathione (GSH)[27], catalase (CAT)[28], superoxide dismutase (SOD)[29], and glutathione-S-transferase (GST)[30] were determined using Bio-diagnostic kits according to the manufacturer's instructions (Giza, Egypt).

Histopathological Examination

Histological sections (4 μm) thick were prepared from paraffin blocks of hepatic tissues fixed in 10% formal saline. Sections were stained with hematoxylin and eosin (H&E)[31] and Picro Sirius Red staining[32].

Liver Collagen Percentage by Morphometric Image Analysis

The principle behind computer-based morphometry is the different staining pattern of cells, nuclei, and fibers following Sirius Red staining[33]. The pictures were analyzed with LIVARQ500 software (Pathology Department of National Research Center).

Statistical Analysis

Values were expressed as means±SE. To evaluate differences between the groups studied, one way analysis of variance (ANOVA) with Duncan post-hoc was used to compare the group means, and $P<0.05$ was considered statistically significant. SPSS, for Windows (version 15.0) was used for the statistical analysis.

RESULTS

Acute Toxicity

While none of the six rats died after oral administration of *S. officinalis* IE at the higher dose (5000 mg/kg), there was evidence of toxicity at the end of experiment, including increased serum ALT and serum AST activities, as well as deviation in the histopathological examination of the liver. Thus, we used the lower dose of 2000 mg/kg as the limiting dose. At this IE dose, there was no mortality and no sign of toxicity in the rats during the experiment (Figure 1). Doses above 2000 mg/kg were then considered to be those that would kill half the animals in a single lethal dose of IE (LD_{50}). The median effective dose (ED_{50}) was selected based on the proposed LD_{50} obtained from the acute toxicity study. This dose was considered one tenth of the proposed LD_{50}-00 mg/kg body weight orally.

Free Radical Scavenging Activity

Radical scavenging activities were estimated by

comparing the percentage inhibition of DPPH radicals by the tested extract and ascorbic acid. The effect of IE on DPPH free radical scavenging activity was examined at various concentrations (10-80 mg/mL) each in three replications. The data are displayed with mean±SE of three replications (Figure 2). The present results demonstrate that IE has *in vitro* antioxidant activity, as indicated by dose dependent inhibition of DPPH radicals ranging from 88.32% to 50.36%.

Serum Biomarkers for Liver Function

Table 1 shows a significant increase (P<0.05) in the serum levels of AST, ALT, total protein, and globulin of BDL rats after all the experimental periods, as compared to the corresponding Sham groups. Significant decreases (P<0.05) in the serum levels of albumin and (A/G) ratio were observed. On the other hand, oral administration of 200 mg/kg body weight of IE resulted in significantly decreased (P<0.05) serum AST 28 d after surgery, ALT after 14 and 28 d, total protein after 7 and 28 d, and globulin after all experimental periods, as compared to the corresponding BDL groups. However, significant increases were observed in serum albumin level and the A/G ratio after all the experimental periods.

Serum Cholestatic Indices

Data recorded in Table 2 represent a significant increase (P<0.05) in the serum levels of ALP, GGT, total bilirubin, direct bilirubin, and indirect bilirubin of BDL rats after all the experimental periods, as compared to the corresponding Sham groups. On the other hand, treatment with IE (200 mg/kg body weight, administered orally) significantly decreased (P<0.05) the serum level ALP (28 d after surgery), GGT (7&28 d), total bilirubin (7 d), direct bilirubin (28 d), and indirect bilirubin at all the experimental periods as compared to the corresponding BDL groups; however, these did not rise to the levels seen in the Sham group.

Determination of Oxidative Stress Parameters

While significant decreases (P<0.05) were observed in liver GST and SOD after all the experimental periods, GSH was significantly reduced

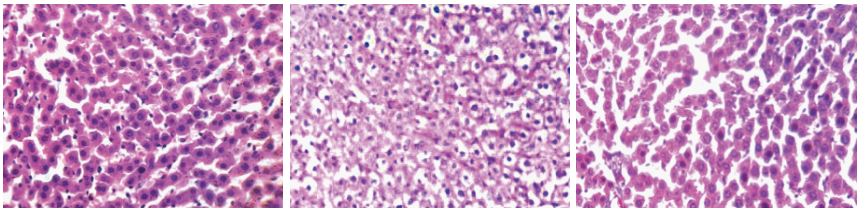

Figure 1. Photomicrograph of rat liver stained with hematoxylin and eosin (40 ×). (A) Control rat. (B) Rat treated with 5000 mg/kg IE. (C) Rat treated with 2000 mg/kg IE.

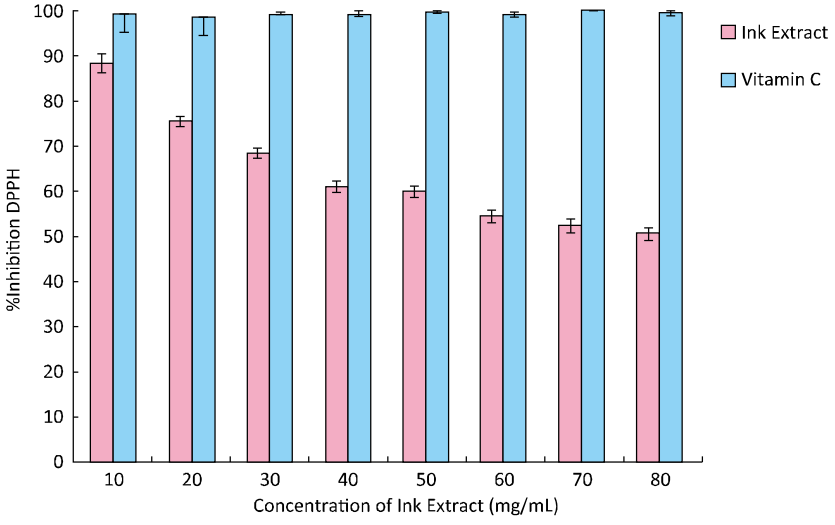

Figure 2. Inhibition of DPPH by *Sepia officinalis* ink extract.

Table 1. Antioxidant Effect of *Sepia officinalis* Ink Extract on Serum Biomarkers for the Liver Function of Bile Duct Ligated Rats

Parameters	Groups	Experimental Period (d)		
		7	14	28
AST (U/mL)	Sham	149.70±9.57^{a}	143.14±9.08^{a}	144.90±8.63^{a}
	BDL	191.55±5.87^{b}	208.25±10.02^{b}	262.85±7.93^{c}
	BDL+Ink	203.65±6.25^{b}	203.65±6.25^{b}	148.15±7.71^{a}
ALT (U/mL)	Sham	55.51±0.99^{a}	54.18±1.2^{a}	55.52±1.1^{a}
	BDL	77.31±5.39^{c}	103.86±2.62^{d}	110.35±5.25^{d}
	BDL+Ink	69.00±6.86^{b,c}	62.65±3.78^{a,b}	58.10±5.08^{a,b}
Total protein (mg/dL)	Sham	4.43±0.46^{a}	3.55±0.5^{a}	4.17±0.51^{a}
	BDL	18.62±0.5^{d}	13.86 ±2.91^{b,c}	15.42±1.89^{c,d}
	BDL+Ink	10.34±0.93^{b}	16.26±2.23^{c,d}	10.27±0.37^{b}
Albumin concentration (g/100 mL)	Sham	5.51±0.32^{b,c}	5.86±0.33^{c}	6.10±0.22^{c}
	BDL	2.81±0.15^{a}	4.5±0.22^{b}	2.63±0.47^{a}
	BDL+Ink	5.10±0.26^{b,c}	7.46±0.76^{d}	5.66±0.41^{b,c}
Globulin concentration (g/100 mL)	Sham	1.27±0.25^{a}	1.72±0.13^{a}	1.50±0.10^{a}
	BDL	15.79±0.50^{e}	11.96±1.42^{d}	12.79±1.45^{d}
	BDL+Ink	5.26±0.68^{b}	8.80±1.50^{c}	4.61±0.14^{b}
A/G ratio	Sham	3.75±0.15^{c}	3.50±0.30^{c}	3.78±.30^{c}
	BDL	0.10±0.01^{a}	0.39±0.04^{a}	0.20±0.02^{a}
	BDL+Ink	1.00±0.07^{b}	0.90±0.06^{b}	1.24±0.11^{b}

Note. Values are given as mean±SE for 6 rats in each group. Values not sharing a common letter superscript are significantly different (P<0.05).

Table 2. Antioxidant Effect of *Sepia officinalis* Ink Extract on Serum Cholestatic Indices of Bile Duct Ligated Rats

Parameters	Groups	Experimental Period (d)		
		7	14	28
ALP (IU/L)	Sham	149.49±12.6^{a}	148.55±12.88^{a}	149.75±12.69^{a}
	BDL	218.29±4^{b}	247.14±4.84^{b}	247.18±3.3^{b}
	BDL+Ink	214.42±22.91^{b}	231.75±9.47^{b}	158.59±21.25^{a}
GGT (U/L)	Sham	19.55±1.22^{a}	19.69±0.83^{a}	16.63±1.19^{a}
	BDL	70.64±7.19^{c,d}	75.04±3.79^{d}	100.75±8.89^{e}
	BDL+Ink	48.40±3.88^{b}	70.64±5.90^{c,d}	56.74±10.10^{b,c}
Total Bilirubin concentration (mg/dL)	Sham	0.50±0.08^{a}	0.48±0.08^{a}	0.47±0.08^{a}
	BDL	8.42±0.67^{c}	5.90±0.38^{c}	8.61±0.72^{c}
	BDL+Ink	6.71±1.39^{b}	5.89±0.12^{b,c}	6.89±0.66^{b,c}
Direct Bilirubin concentration (mg/dL)	Sham	0.19±0.02^{a}	0.19±0.02^{a}	0.19±0.02^{a}
	BDL	3.18±0.4^{b}	2.45±0.31^{b}	2.99±0.17^{b}
	BDL+Ink	3.01±0.55^{b}	2.29±0.4^{b}	0.94±0.14^{a}
indirect Bilirubin concentration (mg/dL)	Sham	0.24±0.02^{a}	0.24±0.02^{a}	0.28±0.2^{a}
	BDL	6.3±0.31^{e}	6.01±0.46^{e}	6.43± 0.99^{e}
	BDL+Ink	3.69±0.15^{c}	1.36±0.09^{b}	5.15±0.37^{d}

Note. Values are given as mean±SE for 6 rats in each group. Values not sharing a common letter superscript are significantly different (P<0.05).

14 and 28 days after surgical procedures (Table 3). While there was no significant change (P>0.05) in liver CAT after all the experimental periods, there was a significant increase (P<0.05) in malondialdehyde (MDA) only after 28 days (Table 3). In contrast, treatment with IE (200 mg/kg body weight, administered orally) resulted in a noteworthy reduction of (P<0.05) liver GSH and GST 14 and 28 after surgical procedures and after 28 days only in liver SOD. The MDA level decreased significantly after all the experimental periods, in comparison to levels in the corresponding BDL groups.

Histopathological Examination

H&E stains (Figure 3) were similar to Picro Sirius Red stains (Figure 4). Sham rats had a normal distribution of collagen, whereas BDL rats demonstrated signs of fibrosis. Fibrosis increased with time, with collagen deposition increasing to maximum value 44.19±2.19 for BDL rats at 28 days. On other hand, groups treated with IE (200 mg/kg body weight, administered orally) showed reduced collagen deposition as compared to BDL groups (Figure 3).

Liver Collagen Percentage by Morphometric Image Analysis

The liver collagen percentage in BDL rats increased significantly (P<0.05) after all the experimental periods when compared to the corresponding percentage in the Sham groups (Table 4). On the other hand, a significant decrease (P<0.05)

Table 3. Antioxidant Effect of *Sepia officinalis* Ink Extract on Oxidative Stress Parameters on Liver of Bile Duct Ligated Rats

Parameters	Groups	Experimental Period (d)		
		7	14	28
MDA (nmol/g tissue)	Sham	8.15±0.57^{b,c}	8.03±0.59^{b,c}	8.00±0.57^{b,c}
	BDL	8.79±0.14^{c}	9.32±0.37^{c}	15.04±0.42^{d}
	BDL+Ink	6.37±5.11^{a}	7.31±0.35^{a,b}	7.37±0.26^{a,b}
GSH (mg/g tissue)	Sham	7.759±0.91^{c,d}	7.74±0.87^{c,d}	7.94±0.89^{c,d}
	BDL	6.65±0.21^{a,b,c}	5.28±0.22^{a,b}	4.77±0.62^{a}
	BDL+Ink	7.11±0.48^{b,c}	8.26±0.9^{cd}	9.49±0.99^{d}
Catalase (U/min)	Sham	2.30±01.56^{a}	2.32±1.52^{a}	2.28±1.28^{a}
	BDL	0.60±0.30^{a}	0.56±0.31^{a}	0.16±0.08^{a}
	BDL+Ink	1.55±0.38^{a}	0.94±0.37^{a}	0.812±0.17^{a}
GST (nmol /min/mg protein)	Sham	0.05±0.003^{c}	0.04±0.008^{b,c}	0.05±0.004^{c}
	BDL	0.04±0.002^{a,b}	0.03±0.002^{a}	0.03±0.006^{a}
	BDL+Ink	0.05±0.005^{b,c}	0.04±0.07^{b,c}	0.043±0.001^{b,c}
SOD (U/g tissue)	Sham	64.68±2.18^{d}	67.22±1.94^{d}	60.43±1.28^{c,d}
	BDL	52.61±4.83^{b,c}	39.66±2.56^{a}	38.54±3.15^{a}
	BDL+Ink	53.39±6.82^{b,c}	46.21±1.39^{a,b}	59.74±3.38^{c,d}

Note. Values are given as mean±SE for 6 rats in each group. Values not sharing a common letter superscript are significantly different (P<0.05).

Table 4. Antioxidant Effect of *Sepia officinalis* Ink Extract on Liver Collagen Percent of Bile Duct Ligated Rats

Item		Experimental Period (d)		
		7	14	28
Groups	Sham	9.65±0.32^{a}	10.27±0.46^{a}	9.81±0.66^{a}
	BDL	20.01±1.98^{c}	38.46±1.28^{e}	44.19±2.19^{f}
	BDL+Ink	15.17±0.89^{b}	22.35±0.36^{c,d}	25.86±1.67^{d}

Note. Values are given as mean±SE for 6 rats in each group. Values not sharing a common letter superscript are significantly different (P<0.05).

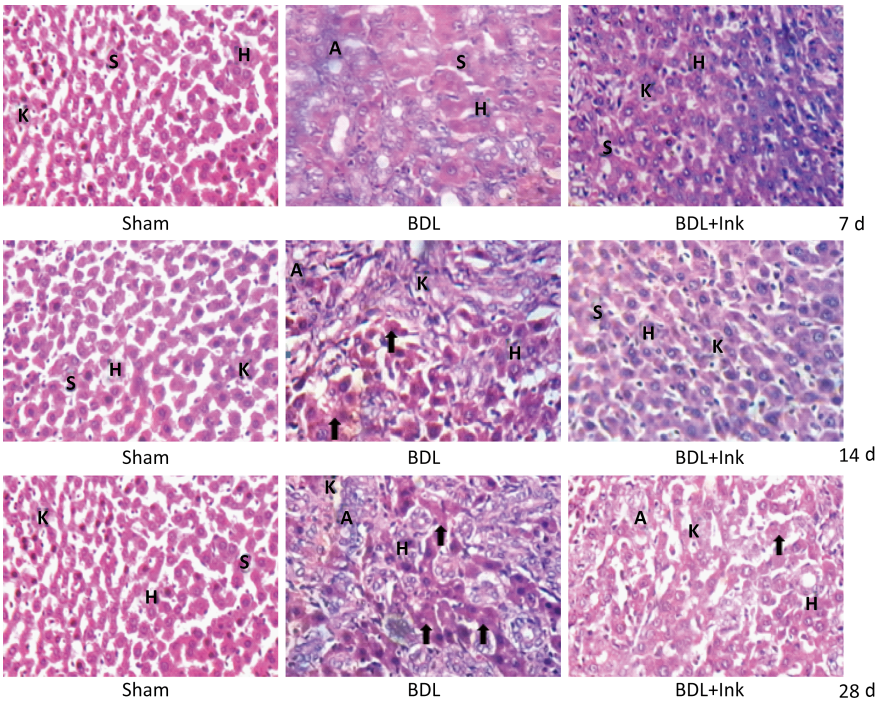

Figure 3. Photomicrograph of rat liver sections. Sham group, showing cords of hepatocytes (H) separated by blood sinusoids (S). Kupffer cells (K). After BDL for three time periods, liver fibrosis indicated by presence of many collagen fibers (arrows). After treatment with IE (200 mg/kg body weight, administered orally) for three time periods, demonstrating the regeneration of liver parenchyma and light fibrosis (H & E, 400 ×).

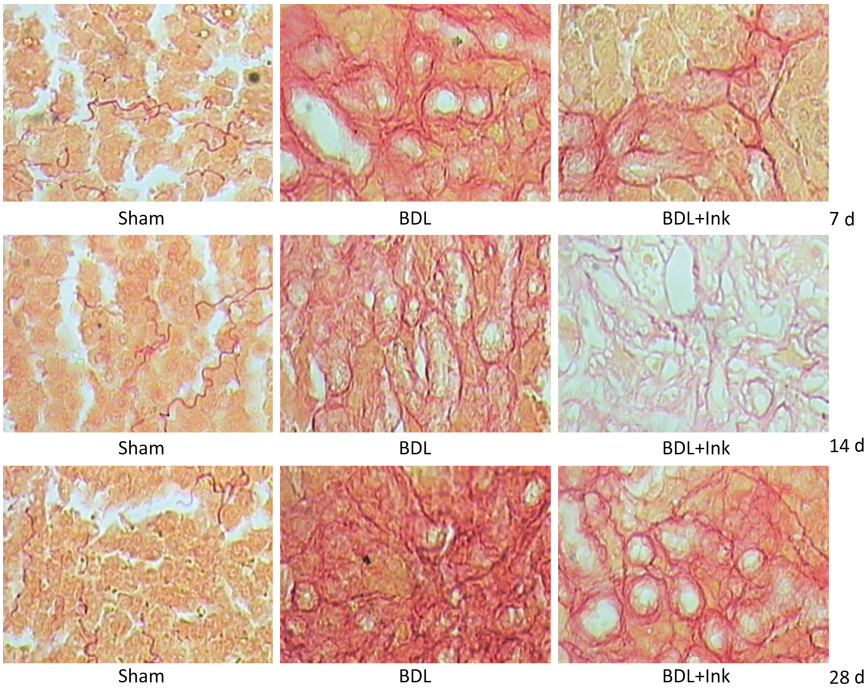

Figure 4. Histopathological examination of liver by Sirius Red staining. Sirius Red staining of the liver from all Sham rats at each time period revealed normal lobular architecture and normal collagen distribution. Sirius Red staining of liver tissue of BDL groups shows extensive collagen deposition and pseudo-lobular formation. The degree of collagen deposition decreased in treated groups (200 ×).

in the percent of liver collagen was observed in groups orally administered 200 mg/kg body weight IE after all the experimental periods, as compared to the corresponding BDL group.

DISCUSSION

Cholestasis is a common pathological feature of numerous liver diseases that results in hepatotoxicity, inflammation, and cirrhosis[34]. In order to cure liver injury, it is important to find natural products have antioxidant compounds capable of ameliorating liver injuries by reducing free radical-mediated tissue damage.

In recent years, the ink secretion of molluskan species was identified as a novel source of bioactive compounds. Sepia ink extract (IE) was able to ameliorate therapeutic injury induced by cyclophosphamide in model animals[35]. Even now, although researchers have studied pharmacological roles of sepia IE for many years, this marine material is still disregarded. A correct understanding and utilization of squid ink may lead to its reuse and reduce the waste of a marine resource.

The Bile Duct Ligation (BDL) model is a typical animal model of secondary biliary disease in animals that features proliferation of bile duct epithelial cells, hepatocellular necrosis, apoptosis, stellate cell activation, and, eventually, the formation of liver fibrosis and cirrhosis, causing fatal damage to the liver[36]. BDL has been associated with cytotoxic bile components such as lipophilic bile acids; that are partly responsible for the plasma membrane damage in BDL models, which leads to further oxidative stress[37].

BDL-induced hepatic damage is characterized by marked biochemical and physiological abnormalities that are emphasized by significant elevation in serum levels of AST and ALT, which are considered to be the most sensitive biomarkers of liver injury[38]. These deleterious changes in liver enzyme levels indicate a loss of tissue integrity with consequent apoptosis and necrosis of hepatocytes[39]. They also reflect the toxic effect of bile acids on the liver and severity of hepatic injury in cholestasis[40]. A general decrease in the activities of serum AST and ALT of rats treated with sepia IE throughout the experiment, as compared to BDL rat groups, indicates the maintenance of function and structure of hepatic cells. Similarly, oral administration of the marine clam (*Gelonia eros*) for 30 d decreased the activities of AST and ALT in rats[41]. The increased level of AST and ALT in our experiment could be considered an indirect sign of inflammation[42].

The liver is the major source of most serum proteins[43]. The site-specific oxidative damage of some susceptible amino acids is regarded as the major cause of metabolic dysfunction during pathogenesis[44]. The present study indicates that the serum total protein concentration of BDL rats increased significantly after all the experimental periods. Elevation of total protein could be attributed to endotoxemia, which frequently follows BDL. In addition, the liver dramatically increased the synthesis of total proteins during the acute-phase response to the stress of the operation[45]. The treatment with sepia IE in the present study resulted in a significant decrease in serum total protein concentration in treated rats.

The liver is the only site of albumin synthesis. Albumin is the most important protein in plasma synthesized by the liver and is a useful indicator of hepatic function[46]. Our study revealed that the serum albumin concentration of BDL rats decreased significantly throughout the experimental periods. This decrease in serum albumin concentration could be related to hepatic dysfunction and decreased protein synthesis[47]. Furthermore, these changes may reflect alterations in metabolism, such as reduced hepatic synthesis associated with the BDL[48]. On the other hand, the serum albumin concentration of rats treated with sepia IE increased significantly after all experimental periods. This effect may be related to the antioxidant properties of sepia IE[49]. Moreover, this increase may be due to enhanced synthesis of proteins and albumin, which works to accelerate the regeneration process, thus affording protection to the liver[50].

Globulins constitute immunoglobulin, produced by B lymphocytes, and β globulins, which are synthesized mainly by hepatocytes[46]. Many investigators have demonstrated that the increase in serum globulin concentration could be attributed to IgA, which is excreted in the bile, and considered as the predominant cell type in liver diseases, and could simply reentered into the serum after BDL[51]. On the other hand, our results showed a significant decrease in serum globulin concentration in rats treated with sepia IE after all the experimental periods. This finding may be due to the protective effect of sepia IE, which decreases the circulating visceral proteins[47].

The A/G ratio is a biochemical parameter utilized to interpret the changes in serum protein

levels that accompany liver diseases[52]. A significant decrease in the A/G ratio of BDL rats, in this study, demonstrates that protein metabolism may have been adversely affected by the inhibition of protein synthesis, such as of albumin, in the liver[53]. On the other hand, our study showed that the A/G ratio of rats treated with sepia IE increased significantly after all the experimental periods. This observation may be attributed to increased protein synthesis. Furthermore, it has been reported that stimulation of protein synthesis may contribute a protective mechanism that accelerates regeneration of hepatocytes[54].

Alkaline phosphatase (ALP) is concentrated in the cells of the bile duct and is often employed to assess the integrity of the plasma membrane of the liver. Elevation of total ALP activity in serum is observed along with increased osteoplastic activity and in hepatobiliary diseases characterized by some degree of cholestasis[55]. The present study discloses that the ALP activity of BDL rats increased significantly after all the experimental periods. This increase occurs due to *de novo* synthesis by liver cells, which is a reliable marker of hepatobiliary dysfunction due to liver damage[56]. In contrast, treatment with sepia IE caused general decreases in serum ALP activity in BDL rats. The lowering of enzyme activity is a definite indication of hepatoprotective action and denotes functional integrity of the hepatic cell membrane[57].

Gamma glutamyl transferase (GGT) is regarded as a component of the cell protection system against oxidative stress. An elevation of GGT is closely related to hepatic injury[58]. Our results revealed that GGT activity in BDL rats increased significantly, which may be due to the increase in hepatic cell membrane fluidity that led to release of the enzyme into circulation[4,59-60]. Alternatively, treatment with sepia IE caused a decrease in serum GGT in BDL rats. This reduction could be attributed to the protective effect of sepia IE on hepatic injury during bile constriction[61].

Testing the levels of serum bilirubin is one of the most sensitive methods employed in the diagnosis of hepatic diseases. Primarily hydrophobic bile salts and unconjugated bilirubin have the most significant toxic effect during cholestasis[56]. Our results show a significant increase in the serum concentrations of total bilirubin, direct bilirubin, and indirect bilirubin in BDL rats after all the experimental periods. This may be due to the degeneration of hepatocytes and blockage of bile ducts caused by the BDL[62]. While treatment with sepia IE causes a general decrease in total bilirubin, direct bilirubin, and indirect bilirubin in the BDL groups, this reduction reflects the role of sepia IE in helping clear bilirubin from circulation[63].

The detergent action of bile salts is responsible for the solubilization of plasma membranes and cell death, which in turn may lead to oxidative stress, oxidation of reduced glutathione (GSH), and lipid peroxidation[64]. There is growing evidence suggesting that considerable impairment of oxidative stress regulation may play an important role in cholestatic liver injury. In addition, accumulation of bile acids leads to oxidative injury and inflammation in hepatocytes. Cholestasis in the BDL model reduces the excretion of bile salts, causing the retention of hydrophobic bile salts within hepatocytes, which leads to apoptosis and necrosis and the destruction of liver parenchyma, which then contributes to a redox imbalance and the formation of reactive oxygen species (ROS). In addition, ROS cause oxidation of cellular proteins and extensive damage to mitochondrial DNA and liver, which affects mitochondrial synthesis[65].

Malondialdehyde (MDA) is a secondary product of oxidative stress formed during lipid peroxidation and is released because of ROS toxicity in rats after BDL[64]. Increased concentrations of MDA reflect the levels of lipid peroxidation in tissues, and MDA is considered a marker of tissue injury. The increased MDA level suggests enhanced lipid peroxidation, leading to tissue damage and failure of the antioxidant defense mechanism to prevent formation of excessive free radicals[66]. However, treatment with sepia IE decreased the level of MDA.

Superoxide dismutase (SOD), an antioxidant, is part of the first line of defense in living cells and works to prevent the production of free radicals. Superfluous free radicals may damage proteins and nucleic acids and can induce lipid peroxidation, which produces large amounts of MDA that injures cells and results in disequilibrium of the internal environment and disease development. Consequently, SOD activity and MDA levels can reflect the antioxidant ability of body. The antioxidant ability of sepia IE was discovered. Melanin obtained from squid ink, like superoxide dismutase, has been reported to catalyze O_2^- to H_2O_2, thus avoiding the free radical chain reaction triggered by O_2^-[67]. Sepia IE dose-dependently elevated SOD activity in the livers and kidneys of mice[68].

Accumulation of bile acids and inflammatory

cells in liver tissue causes free radical production during biliary obstruction. Several lines of evidence have suggested the important role of oxidative stress in the etiopathogenesis of liver fibrosis[56]. Chronic cholestasis increased the concentration of biliary acids in hepatocytes, which induced mitochondrial toxicity and resulted in the overproduction of ROS[69].

Reduced glutathione (GSH) is considered an important cellular protectant against reactive metabolites in several cells by serving as a substrate for glutathione peroxidase[70]. In addition, GSH is an important constituent of protective intracellular mechanisms against various noxious stimuli, including oxidative stress. Reduced GSH, the main component of the endogenous non-protein sulfhydryl pool, is known to be a major low molecular weight scavenger of free radicals in the cytoplasm[71]. It has been proposed that antioxidants, which maintain the concentration of reduced GSH, may restore the cellular defense mechanisms and block lipid peroxidation, thus protecting against oxidative tissue damage[72].

Catalase (CAT) is one of the important enzymes in the supportive team of defense against reactive oxygen species (ROS). Catalase is a hemoprotein, containing four heme groups, that catalyzes the decomposition of H_2O_2 to water and O_2 and, thus, protects the cell from oxidative damage by H_2O_2 and OH[73]. The present study reports that the GSH and CAT levels of BDL rats decrease significantly as compared to levels in the corresponding Sham groups. However, the levels of both increase significantly after treatment with sepia IE.

The current results demonstrate that sepia IE diminishes oxidative stress biomarkers and inhibits lipid peroxidation, SOD activity, and GSH levels. These actions are attributed mainly to sepia IE, which is a multifunctional marine bioactive material that possesses anti-oxidant, anti-radiation, anti-retroviral, and anti-bacterial properties, as many lines of evidence have indicated[74].

Liver fibrosis after BDL injury was characterized morphologically. Hepatic fibrosis results from chronic injury due to various causes and ultimately leads to the irreversible and massive accumulation of extracellular matrix (ECM) proteins[75]. The increased deposition of collagen is one of the major pathologic processes associated with chronic hepatic fibrosis. Liver fibrosis is the pathologic result of ongoing chronic inflammatory liver diseases and is characterized by hepatic stellate cell (HSC) proliferation and differentiation into myofibroblast-like cells, which deposit ECM and collagen. Quiescent HSCs are vitamin A-storing cells found in the space of Disse, and they account for approximately 15% of the total number of liver cells[76]. The activation of HSCs is mediated by reactive oxygen, as well as other factors that are released from damaged hepatocytes and activated Kupffer cells. Activated HSCs produce large amounts of ECM components, such as laminin and collagen, in an accelerated fashion, resulting in fibrotic change of the liver. The number of HSCs increased in all animal models of chronic liver disease. The present study showed that BDL rats exhibited signs of fibrosis that increased over time, with collagen deposition increasing to the maximum value for BDL rats at 28 days. Treatment with sepia IE reduced collagen deposition as compared to that in BDL groups, suggesting that the liver was repaired. Mouse models of liver cirrhosis induced by CCl_4 assess fibrosis, regenerative nodules, and deposition of collagen[77].

The morphological features of cholestasis depend on its severity, duration, and underlying cause. The ducts themselves dilate and are abundantly filled with bile. Droplets of bile pigments can accumulate within hepatocytes. In BDL, the portal tracts swell because of edema. BDL induces proliferation of the duct epithelial cells, causing looping and reduplication of ducts in a process termed bile duct proliferation. Backpressure during BDL fibrosis increases around ducts and extends between adjacent portal tracts, subsequently leading to the death of hepatocytes and ultimately to liver fibrosis and cirrhosis due to bile accumulation[78]. In this work, histopathological examination of liver tissue revealed damage to liver cells that was confirmed by biochemical analyses and observation of oxidative stress parameters. Furthermore, oxidative stress is known to aggravate liver fibrosis via HSC activation, and lipid peroxidation stimulates transcription of the collagen gene[56].

In conclusion, the results of the present study indicate that oral administration of sepia IE at the tested doses attenuates liver fibrosis associated with extrahepatic cholestasis induced by BDL through antioxidant action. In addition, our results reveal the presence of considerable amounts of antioxidants in sepia IE, which provides evidence for its hepatoprotective effect and antioxidant capacity, as shown by significant reduction of hepatic enzymes, bilirubin levels, and oxidative stress in BDL-rats

treated with sepia IE in comparison with untreated BDL groups throughout all the experiment periods.

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