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Selenium Attenuates Cholestasis-Induced Liver Injury and Fibrosis by Alleviating Liver Oxidative Stress and Inflammation in Rats

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Keywords

- Cholestasis
- Selenium
- Ooxidative stress
- Inflammation
- Fibrosis

Abstract

Background: Oxidative stress and inflammation are primarily implicated in the development and progression of liver injury during cholestasis. Selenium, a known essential antioxidant trace element, was found to provide a remarkable antioxidant and anti-inflammatory effects on various diseases. **Aim**: This study was planned to evaluate the possible protective effect of selenium supplementation in a rat model of chronic cholestasis. **Design**: Experimental study. **Methods**: This study was carried out on adult male rats allocated randomly into sham, 4 weeks bile duct ligated (BDL), and BDL-selenium treated (BDL-Se) groups. Sodium selenite was given by gavage daily, in a dose of 100 µg/kg for 6 weeks, starting 2 weeks before the BDL. **Results**: BDL group presented a significant increase in serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and liver levels of malondialdehyde (MDA), tumor necrosis factor alpha (TNF-α), and transforming growth factor beta 1(TGF-β1), associated with a significant decrease in serum levels of total proteins (TP) compared to sham group . Selenium supplementation significantly lowered serum levels of AST, ALT, ALP, and liver levels of MDA, TNF-α, and TGF-β1, along with a significant increase in serum TP in BDL-Se group versus BDL rats. Histological analysis of liver showed a significant attenuation of the inflammatory score and a significant decrease in the percentage area of collagen deposition in BDL-Se group versus BDL rats. **Conclusion**: Selenium supplementation reduces liver injury and improves liver functions in experimental cholestasis probably by its antioxidant and anti-inflammatory activities, which further alleviate the liver fibrosis.

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INTRODUCTION

Extrahepatic cholestasis is caused by structural abnormalities of the biliary tract including obstruction of bile duct and gallbladder. Main contributing causes leading to extrahepatic cholestasis are cholelithiasis, cancer head of pancreas and biliary atresia. The bile stasis caused by the inadequate bile flow results n accumulation of bile constituents, including bilirubin, bile acids and lipids in the liver[1]. Several studies have suggested oxidative stress and inflammation as primary mechanisms behind early liver injury associated with cholestasis[2]. Excessive hydrophobic bile acids produce hepatocellular injury followed by neutrophils recruitment and infiltration which attack the toxic bile acids stressed hepatocytes via reactive oxygen species (ROS) production[3]. Over-production of ROS has been shown to induce cell damage and to magnify the inflammatory process by enhancing TNF-α signaling pathway, and IL-6 mRNA expression in chronic liver diseases[4]. Moreover, oxidative stress was found to activate TGF-β1, a potent profibrotic cytokine, which promotes the proliferation of myofibroblasts[5], the fundamental effector cells of fibrogenesis[6].

Selenium, an essential trace element, is of great importance in the medical research field and plays an important role in scavenging free radical damage to cells[7]. Selenium supplementation provided a cardioprotective, anti-inflammatory and antioxidant effect in a rat model of myocardial infarction[8], and attenuated renal injury in a rat model of renal ischemia/reperfusion[9]. Thus, a therapeutic strategy by selenium might increase the antioxidant capacity of liver cells, ameliorating the inflammatory and the fibrotic processes and afford some protection in chronic cholestasis. The present study was designed to evaluate the possible protective effect of selenium supplementation in a rat model of chronic extrahepatic cholestasis induced by bile duct ligation. The underlying mechanisms will also be analyzed.

Materials and methods:

Animals

Eighty adult male albino rats, weighing 160-290 g were purchased from Experimental Animal Farm, Giza, Egypt. Rats were kept in animal cages and maintained under suitable ventilation, temperature of 22-25˚C, 12 hours light/dark cycle and free access to food and water in the Medical Ain Shams Research Institute (MASRI) animal house. Rats were kept for 7 days for acclimation before starting the experiment. All experimental procedures were carried out according to the guidelines of FMASU, REC (Faculty of Medicine, Ain Shams University, Research Ethics Committee, Cairo, Egypt. FWA 00017585 [MS 16/2020].

Experimental design

Rats were allocated randomly into 3 groups: (1) Sham-operated control group (Sham, n=13): Rats in this group were subjected to all surgical procedure without bile duct ligation and received distilled water; (2) Bile duct -ligated rats (BDL, n=11): Rats in this group were subjected to bile duct ligation for 4 weeks and received distilled water; (3) Bile duct ligated-selenium rats (BDL-Se, n=13): Rats in this group underwent bile duct ligation and received selenium for 6 weeks, starting 2 weeks before the operation. Sodium selenite (Na2O3Se, Sigma, St. Louis, Missouri,

USA) was dissolved in distilled water and given by gavage in a dose of 100 µg /Kg / day[10].

Surgical procedure:

Common bile duct ligation, for 4 weeks, was carried out according to the method described by Tag et al.[11]. A midline abdominal incision was made and the peritoneal cavity was opened under complete sterile conditions. The liver was lifted so that the hilar side and the common bile duct were exposed. Portal pedicle dissection was performed and the common bile duct was double ligated with 2/0 silk suture. The peritoneal cavity was rinsed with 0.9% NaCl solution, and the abdominal organs were placed to their positions and the incision was closed. The mortality rate was 13.3% in sham-operated group, and 63% in all rats exposed to bile duct ligation surgery.

At the end of the experimental period, the overnight fasted rats were weighed (BW), anesthetized, and blood samples were collected from abdominal aorta in plastic tubes. Blood was allowed to coagulate at room temperature, then centrifuged at 3000 rpm. for15 min. Serum was collected and stored at -80˚C, till used for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total proteins (TP). Both liver and spleen were excised and weighed. Liver weight (LW) and spleen weight (SW) were used to determine hepatosomatic and splenosomatic indices according to the following formulas[12]:

Hepatosomatic index= $\frac{LW(g)x100}{BW(g)}$ and Splenosomatic index= $\frac{SW(g)x100}{BW(g)}$

Liver tissue samples were stored at -80 ºC until used for tissue assays of oxidative stress marker, malondialdehyde (MDA); the proinflammatory marker, tumor necrosis factor alpha (TNF-α); and the profibrotic marker, transforming growth factor beta-1(TGF-β_1). Liver tissue samples were also fixed in 10% formalin for histological analysis.

Biochemical analysis:

Serum AST and ALT were determined using kits supplied by Biodiagnostic (Giza, Egypt) according to the method described by Reitman and Frankel (1957) [13]. Serum ALP was measured using kits supplied by Biodiagnostic (Giza, Egypt) according to the method reported by Belfield and Goldberg (1971) [14]. Serum TP was measured using kits supplied by Reactivos (GPL, España) according to the method reported by Burtis and Ashwood (1999) [15].

Measurement of MDA, TNF-α and TGF-β_1 in liver tissue

MDA was measured by using malondialdehyde Kit supplied by Cell Biolabs (Inc, San Dieago, USA), according to the method described by Armstrong and Browne (1994) [16]. TNF-α was determined using Rat Tumor Necrosis Factor Alpha ELISA kit, purchased from Wuhan Fine Biotech Co. (China), according to the manufacturer's instructions. TGF-β1 was determined by quantitative sandwich ELISA method according to Sporn et al. (1986) [17], by using rat TGF-β1 ELISA kit, supplied by Wuhan Fine Biotech Co. (China).

Histological examination:

Liver tissues were fixed in 10% formalin, processed and paraffin sections were prepared. The sections were stained by Hematoxylin & Eosin (H&E) and Masson's trichrome (MTC). All liver tissue sections were evaluated blindly. In each

group, liver sections were prepared from five randomly selected rats. Then, three different non-overlapping fields were examined from each section. Accordingly, 15 fields were used for grading of inflammation and for determining the percentage area of fibrosis in the individual group. The liver inflammation was graded into 5 grades according to Scheuer (1991) [18]. The inflammatory score was determined as grade 0: normal (no portal/periportal inflammation or lobular necrosis); grade 1: minimal (portal inflammation without lobular necrosis), grade 2: mild (periportal inflammation with lobular focal or unicellular necrosis), grade 3: moderate (periportal inflammation with more extensive lobular necrosis) and grade 4: severe (periportal inflammation with lobular bridging necrosis). The percentage area of collagen fibers was measured by Digital Image Analysis System (Carl Zeiss Axiovision Product Suite DVD 30), using Leica Quin 500C Image Analyzer Computer System (Leica Imaging System Ltd., Cambridge, England).

Statistical analysis:

One-way analysis of variance (ANOVA) was used to determine the differences between groups. Least significant difference test was used to find significant intergroup differences. Data of the histological inflammatory grades were presented as median and range, and the statistical analysis was determined using Kruskal–Wallis test. P values ≤0.05 were considered statistically significant. SPSS windows version 20 (SPSS Inc., Chicago, IL, USA) was used in the analysis.

Results:

Changes in hepatosomatic and splenosomatic indices:

Hepatosomatic and splenosomatic indices were significantly increased in BDL rats compared to sham-operated group. In the selenium supplemented group, these indices were not significantly changed compared to BDL rats (Table 1).

Changes in serum enzyme markers of liver injury and total proteins:

The serum levels of AST, ALT, and ALP were significantly increased in BDL rats compared to sham-operated group. All these parameters were significantly decreased by selenium supplementation in BDL-Se group compared to BDL rats, achieving levels comparable to those of the sham-operated controls. The serum level of TP was significantly decreased in BDL group versus sham-operated controls. Selenium supplementation significantly increased the TP in BDL-Se group compared to BDL rats, however, their levels were still significantly lower than their corresponding values in the sham-operated group (Table 1).

Changes in liver tissue levels of malondialdehyde, TNF-α, and TGF-β1:

BDL rats showed significantly increased levels of the oxidative stress marker, MDA, in liver tissue compared to sham-operated group. Selenium supplementation decreased MDA significantly in BDL-Se group in comparison to BDL rats, reaching even lower levels than that in sham group, although statistically it does not reach the level of significance (Figure 1).

Table 1: Changes in hepatosomatic index (HI), splenosomatic index (SI), serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total proteins (TP) in the three study groups.

	Sham (13)	***BDL (11)***	***BDL-Se (13)***
HI (%)	*2.59 ±0.14*	*4.06 ±0.25[a]*	*3.52 ±0.40[a]*
SI (%)	*0.22 ±0.02*	*0.41 ±0.06[a]*	*0.50 ±0.07[a]*
AST (U/mL)	*7.58 ±0.78*	*67.07 ±6.78[a]*	*10.41 ±0.95 [b]*
ALT (U/mL)	*13.78 ±2.28*	*108.85 ±14.78[a]*	*13.50 ±1.43[b]*
ALP (IU/L)	*13.70 ±0.62*	*184.37 ±20.26[a]*	*13.53 ±0.92[b]*
TP (g/dL)	*7.71 ±0.23*	*4.42 ±0.31[a]*	*6.61 ±0.34[ab]*

Data are expressed as mean ±SEM.
In parenthesis is the number of rats in each group.
a: significance of difference from sham group, calculated by LSD at $P \leq 0.05$.
b: significance of difference from BDL group, calculated by LSD at $P \leq 0.05$.
BDL: bile duct-ligated group, BDL-Se: bile duct ligated-selenium group.

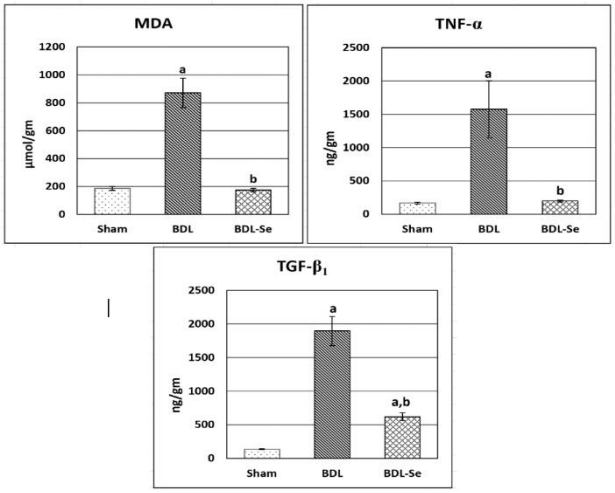


Figure 1: Changes in liver tissue levels of malondialdehyde (MDA), tumor necrosis factor alpha (TNF-α) and transforming growth factor beta-1 (TGF-β_1) in the three study groups.
Data are expressed as mean ±SEM.
a: significance of difference from sham group, calculated by LSD at $P \leq 0.05$.
b: significance of difference from BDL group, calculated by LSD at $P \leq 0.05$.
Sham-operated group (n=13), BDL: bile duct-ligated group (n=11), BDL-Se: bile duct ligated-selenium group (n=13).

The proinflammatory marker, TNF-α, and the profibrotic marker, TGF-β1 were significantly elevated in BDL group compared to sham-operated controls, whereas selenium supplementation significantly decreased them in BDL-Se group versus BDL rats. In comparison to sham-operated controls, TNF-α was not significantly different in BDL-Se group; while TGF-β1 showed significantly higher values (Figure 1).

Histopathological changes in liver tissue:

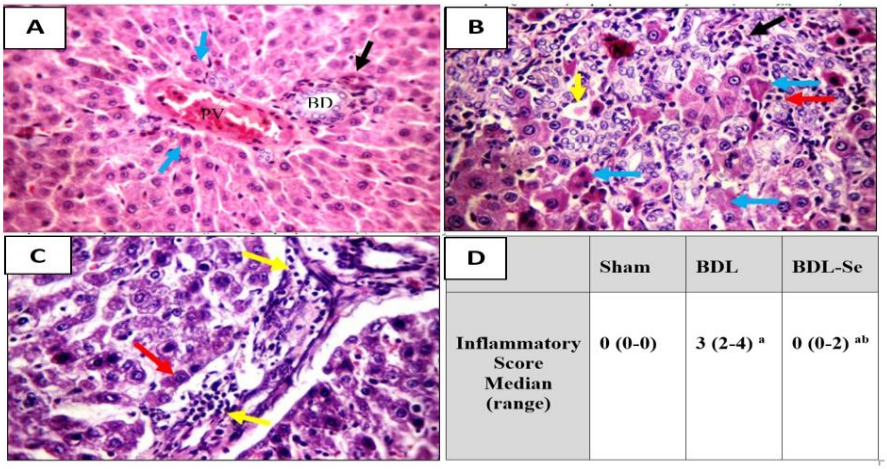
Hematoxylin and eosin stained liver sections:

Sham operated group liver sections showed normal liver architecture of classic hepatic lobules. BDL group showed loss of normal classic hepatic architecture with markedly expanded edematous portal tracts, marked portal and peri-portal inflammatory infiltrate and fibrous bands connecting central vein with portal tract. BDL-Se group showed expanded portal tracts with dilated portal veins and proliferating bile ducts with normal epithelial lining, mild portal inflammatory infiltrate (Figure 2). Concerning the grading of the histological markers of inflammation, the inflammatory score was significantly higher in BDL group compared to sham operated controls. Selenium treatment significantly attenuated the inflammatory score in BDL-Se group compared to BDL rats, though it was still significantly higher in

BDL-Se group versus sham operated controls (Figure 2).

Masson's trichrome stained liver sections:

Sham operated group liver sections showed average collagen distribution around central veins and in portal tracts, with no detected fibrosis. In the BDL group liver sections, fibrous bands connecting portal tracts (portal-portal septa) and excess collagen with nodular formation, expressing definite cirrhosis were observed. Regarding the BDL-Se group, liver sections showed fewer collagen fibers deposition with nodular formation (Figure 3). The percentage area of collagen deposition showed a significant increase in the BDL group compared to sham-operated group. Selenium supplementation resulted in significant decrease in the percentage area of collagen deposition in the BDL-Se group versus BDL rats; however, it was still significantly higher than its matching value in sham-operated controls (Figure 3).



	Sham	BDL	BDL-Se
Inflammatory Score Median (range)	0 (0-0)	3 (2-4) [a]	0 (0-2) [ab]

Figure 2: Photomicrograph of liver sections (H&E x400). [A]: Sham operated control group shows average portal tract with average portal vein (PV), average bile duct (BD), average hepatic artery (black arrow), and average hepatocytes in the interface area (blue arrows). [B]: Bile duct ligated group shows moderate portal (black arrow) and peri-portal inflammatory infiltrate (red arrow), markedly apoptotic hepatocytes in the peri-portal area (blue arrows), and bile stasis (yellow arrow). [C]: Bile duct ligated-selenium group shows portal tract with mild portal inflammatory infiltrate (yellow arrows), and average hepatocytes in the interface area (red arrow). [D]: Changes in the inflammatory score in the three study groups.
a: significance of difference from sham group, calculated by Kruskal-Wallis H test at $P \leq 0.05$.
b: significance of difference from BDL group, calculated by Kruskal-Wallis H test at $P \leq 0.05$.

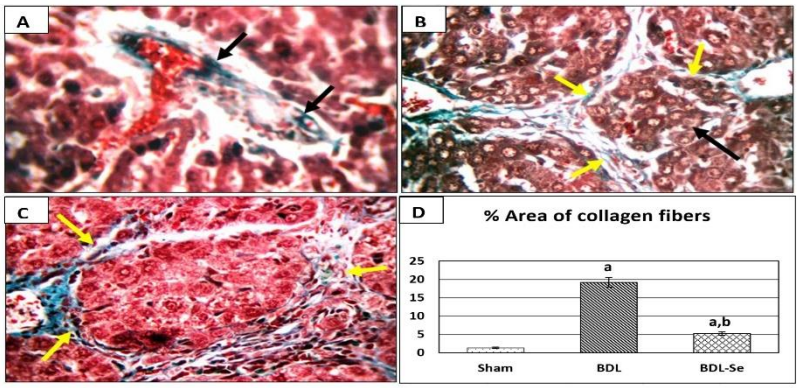


Figure 3: Photomicrograph of liver sections (Masson trichrome x 400). [A] Sham operated control shows average collagen distribution in portal tract (black arrows). [B] Bile duct ligated (BDL) group shows excess collagen (yellow arrows) with nodular formation (black arrow). [C] Bile duct ligated-selenium group shows markedly decreased amount of collagen fibers deposition compared to (BDL) liver section with nodular formation (yellow arrows). [D] Changes in the percentage area of collagen fibers in the three study groups.
a: significance of difference from sham group, calculated by LSD at $P \leq 0.05$.
b: significance of difference from BDL group, calculated by LSD at $P \leq 0.05$.

Discussion:

The present study aimed to highlight the potential protective effect of selenium administration on cholestatic liver injury and its underlying mechanisms. Cholestasis was induced in rats by bile duct ligation, an established model for cholestatic liver injury in rodents[11].

The results of the present study showed that bile duct ligation for 4 weeks was able to induce liver cell injury as evidenced by the significant increase in serum liver enzyme levels, namely AST, ALT and ALP, well known markers of hepatocellular damage[19]. These data are in agreement with Tag et al., (2015) [11], and Wu et al. (2017) [20] and could be explained by bile acids accumulation in the biliary canaliculi, which were reported to act as detergents, thus damage the plasma membrane of hepatocytes, releasing liver enzymes in the blood[21].

Additionally, the cholestasis-induced liver injury, herein, was associated with deterioration of liver function as manifested by the significant decrease in total serum protein level (TP) in the BDL group in comparison to the sham-operated group. This observation is consistent with Li et al. (2020) [22].

The selenium treatment, in the present study, has the ability to correct disruption in liver enzymes returning them to normal values, and to attenuate the impairment of liver function, as demonstrated by the significant decrease in ALT, AST, and ALP along with a significant increase in TP in BDL-Se group compared to BDL rats, all achieved levels comparable to those of the sham-operated controls except TP which was still significantly lower than control value. These results confirm the finding of a previous study, indicated that selenium could alleviate the dysregulation of liver marker enzyme (ALT and AST) activity and protein (albumin and TP) levels in the serum of cyclophosphamide treated geese[22].

This protective effect of selenium could be ascribed to its antioxidant effect as evidenced by the ability of selenium treatment to significantly reverse the elevated MDA after bile duct ligation in BDL-Se group versus BDL rats; the level of MDA becomes even less than that in sham-operated control rats, although statistically it does not reach the level of significance. This assumption agrees with that of Fatima and Mahboob (2013) [23], who observed that sodium selenite supplementation markedly reduced total bilirubin and ALT activity and restored the antioxidant enzymes (SOD and GPx) , MDA and catalase activity to normal in a rat model of thioacetamide induced liver cirrhosis. Recently, Li et al. (2020) [22] found that selenium treatment significantly inhibited the increase in the MDA level and the decrease in the GPx, catalase, and SOD activity in cyclophosphamide-induced liver injury, relating the reduction of hepatocyte necrosis to the inhibition of oxidative stress.

Additionally, TNF-α, a chief proinflammatory cytokine that triggers inflammation, has been implicated in liver damage and hepatocyte apoptosis in different liver diseases including cholestatic liver diseases[24] [25]. Accumulation of bile acids has been shown to increase neutrophil infiltration, enhancing the development of inflammation in liver tissues and

promoting liver damage via releasing of inflammatory cytokines such as, IL-1, IL-6 and TNF-α [26 27 28]. In line with these reports, BDL rats in the present study showed a significant high liver levels of TNF-α associated with portal inflammatory infiltrate composed of macrophages with scattered eosinophils, and scattered apoptotic hepatocytes, denoting the development of a significant local inflammatory response in cholestasis. Selenium treatment, herein, was associated with a significant amelioration of liver levels of TNF-α in BDL-Se group compared to BDL rats, approaching levels comparable to those of sham-operated controls. Moreover, H & E stained liver sections of BDL-Se rats displayed a reduction of the histological inflammatory markers with a significant decrease in the inflammatory score compared to BDL group, reflecting an anti-inflammatory effect of selenium.

The ability of selenium to provide a hepatoprotective effect via inhibiting the liver inflammatory process has been documented by recent studies in different animal models of liver injury[29 30 31 32]. Earlier, TNF-α knockout was found to prevent CCL4-induced liver damage in mice[33]. Therefore, the attenuation of liver levels of TNF- α as well as the liver inflammatory reaction produced by selenium treatment, in the present study, might afford an additional hepatoprotective mechanism in association with the antioxidant activity.

Furthermore, the current results revealed a significant increase in liver levels of TGF-β1 accompanied by significant increase in the percentage area of collagen fibers deposition (determined by the percentage of Masson's trichrome expression in liver sections), and excess collagen with nodular formation, expressing definite liver cirrhosis in BDL group compared to sham-operated controls.

TGF-β1, a cytokine that has a key role in liver fibrogenesis[34], was found to be secreted by activated hepatic stellate cells, promoting collagen I, alpha- smooth muscle actin, and tissue inhibitor of metalloproteinases expressions, leading to excess extracellular matrix (ECM) production and liver fibrosis[35]. Also, TGF-β1 expression has been proved to be induced 5 times and 7.5 times after 8 and 30 days BDL, respectively, when compared with control animals[36].

An important observation, in the present study, was the ability of selenium to ameliorate fibrosis induced by bile duct ligation as demonstrated by the significant decrease in liver levels of TGF-β1 and in the percentage area of collagen fibers deposition in livers, in BDL-Se group versus BDL rats. These results are in accord with those of Liu et al. (2015); El Shater and Ali (2019) and Liu et al. (2019) [37 38 39], and notify an antifibrotic activity of selenium treatment in liver cholestasis.

Several previous studies reported that selenium supplementation in murine model of fibrotic liver disease caused significant decrease in liver profibrotic and inflammatory markers[38 40]. Besides, Lu et al. (2017) [41] reported that selenium-enriched ziyang green tea polysaccharide treatment induced a sensible repression on TGF-β1-induced type I collagen synthesis compared to those in untreated controls. Moreover, Ding et al. (2010) [40] clarified that the cause of the antifibrotic effect of selenium is due to the decrease in collagen-producing stellate cells number and subsequent

amelioration in fibrosis resulting after CCl4 treatment.

The detrimental effect of interplay between oxidative stress, and inflammation on liver fibrosis, has been postulated in the present study; as cholestasis initiates liver oxidative stress, and inflammation that interact together, promoting the development of liver fibrosis and impaired liver functions. Selenium treatment, herein, disrupts such interaction, alleviating liver fibrosis and providing a considerable hepatoprotective effect, probably via its antioxidant as well as anti-inflammatory activities.

Regarding the liver and spleen weight and indices in BDL group, they displayed marked increase, denoting hepatosplenomegaly as a result of hepatic inflammatory process and the subsequent portal hypertension. Similar results were detected by Garrido et al. (2017) [42].

Surprisingly, after treatment with selenium, liver weight, hepatosomatic index, spleen weight, and splenosomatic index were not significantly changed in BDL-Se group versus BDL rats. In contrast, a reduction in the liver weight and relative liver weight was observed in TAA-selenium treated group as compared to control[23]. Elsewhere, Li et al. (2020) [22] listed that liver weight in the selenium treated group showed marked decrease compared to the cirrhotic group, in a study performed on geese. The discrepancy between the present results and the previous ones, might be explained by the differences in the animals, model of liver injury, dose, and/or duration of selenium administration used in these studies.

In conclusion, the data presented, herein, provide evidence that selenium treatment attenuates liver injury and improves liver functions in experimental cholestasis induced by bile duct ligation in rats. Such hepatoprotective effect might be probably mediated by its antioxidant, and anti-inflammatory activities via decreasing the levels of MDA and TNF-α locally in liver tissues, which further alleviate the liver fibrosis, inhibiting TGF-β1 generation. Selenium treatment can be used as a potential candidate regimen for attenuating the progression of cholestatic liver diseases; however, toxicological and clinical studies are needed prior to recommendation for humans.

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Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

Contributors: FM Lebda and SM El Agaty put the idea and design of the work, NA Nassef and MA Youssef performed the experiment. All authors participated in analysis of data, writing and revising the manuscript in its final form.

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