The Egyptian Journal of Hospital Medicine (October 2016) Vol. 65, Page 583- 591

Histopathological And Immunohistochemical Evaluation of Antifibrogenic Effect of Grape Seed Extract on CCl4-Induced Model of Hepatic Fibrosis

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ABSTRACT

Introduction: Liver fibrosis represent a worldwide challenge of clinical importance, results from chronic damage of liver, and evidenced by build up of excessive extracellular matrix proteins.. The present study was carried out to evaluate the antifibrogenic effect of grape seed extract (GSE) against hepatic fibrosis induced by CCl4 in mice.

Experimental Design: forty adult male albino mice were divided into four equal groups; first (control) in which mice were injected IP with olive oil as vehicle. In the second group (GSE) mice were received GSE orally at a dose of 200mg/kg/day for 8 weeks while in the third group (CCl4) mice were injected IP with CCl4 (0.4ml/kg / twice weekly) for 8 weeks . In the fourth (GSE+ CCl4) group mice were injected IP with CCl4 and co-treated with GSE orally as in previous treated-groups. At the end of the experiment, animals were sacrificed and blood samples and liver tissue specimens were collected.

Results: the examined liver of CCl4-intoxicated group revealed marked hepatic fibrotic lesions confirmed by Masson's trichrome stain and associated with the presence of intensely stained α -SMA-positive hepatic stellate cells (HSCs) in entire of the hepatic lobules and in the vicinity of bridging fibrotic septa. Hepatic degeneration and necrosis were also seen. This hepatic damage was associated with significant increases in AST and ALT activities with low albumin levels and hypoproteinemia. Co-administration of GSE with CCl4 improved the microscopic picture of liver where scanty fibrotic lesions and mild degeneration of some hepatic cells were recorded. Less intensely stain
ed α -SMA-immunopositive cells were observed. Serum AST , ALT, albumin and total protein values were more or less within the ranges of these parameters in the control non-intoxicated group.

Conclusion: GSE has potent antifibrogenic effect on CCl4-induced hepatic fibrosis by inhibiting HSCs activation, decreasing collagen synthesis and improving hepatic regenerative capability through its powerful antioxidant and anti-inflammatory properties.

Keywords: Liver fibrosis, Grape seed extract, Histopathology, immunohistochemistry, serum Biochemical analysis.

INTRODUCTION

Hepatic fibrosis is a public health problem commonly accompanied with significant morbidity and mortality rates [1]. Liver fibrosis arises from improper tissue repair through connective tissue deposition that occurs in chronic liver injuries such as chronic viral hepatitis, autoimmune diseases and metabolic disorders [2]. Pathogenesis of liver fibrosis is complex process, as hepatic injury persists, regenerative response of parenchymal cells fails and then hepatocytes are replaced by abundant extracellular matrix (ECM) [3]. The main sources of these ECM are hepatic stellate cells[4]. Therefore, activation of hepatic stellate cells (HSCs) considered as an important step in liver fibrogenesis and leads to progressive accumulation of ECM, which finally leads to liver fibrosis [5]. In addition, activated HSCs increase the production of alpha-smooth muscle actin (α-SMA) and collagen [6].

Liver fibrosis frequently progresses to cirrhosis, hepatic failure, portal hypertension, and hepatocellular carcinoma [7]. In advanced stages of hepatic fibrosis, liver transplantation is the only treatment for patients. Therefore, new strategies for anti-fibrotic therapy are essential and using of natural by products including medicinal plants is one of these strategies.

Received: 11/8/2016
Accepted: 23/8/2016

DOI : 10.12816/0033768

Grape seed is one of these natural products, which had many therapeutic effects. It contains numerous active components such as flavonoids and polyphenols with powerful antioxidant and free radical scavenging capacity [8]. This polyphenol in grape seeds inhibits cancer cell proliferation [9, 10]. While, flavonoids had the ability to increase intracellular vitamin C levels, decrease capillary permeability and fragility [11, 12].

Procyanidines, is the major biochemically active component of GSE which had free radical scavenging activity [13]. Beyond their free radical scavenging and antioxidant activity procyanidines have anti-inflammatory, anti-allergic and anti-tumor activities[14,15,16]. GSE is considered as multi-organ protective agent, including hepatoprotective [17,18] nephroprotective effects[19] and cardioprotective effects [20]. Also, GSE reduce fibrogenic effect of silica-induced pulmonary fibrosis by decreasing of the oxidative stress [21].

Therefore, the current study was performed to investigate the antifibrogenic effect of GSE against CCl4-induced hepatic fibrosis through microscopic examination of the liver and assessment of some fibrosis related indices including α-SMA and serum biochemical markers.

MATERIALS AND METHODS

Animals

Apparently healthy adult male albino mice, weighing about 35-45g were taken from Animal house at college of Pharmacy, Aljouf University, KSA. Mice were kept in standard animal cages with 12 hours light and dark cycle, feed on standard ration and provided water *ad libitum*. These mice were adapted to laboratory conditions for one week before the beginning of the experiment. All animals received human care and the study protocol was performed in accordance with the recommendations for the proper care and use of laboratory animals stated by National Committee of Bioethics (NCBE) and Aljouf University Bioethics Committee Guidelines.

Chemicals:

Carbon tetrachloride (99.9%) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Serum AST, ALT, albumin and total protein kits were obtained from ELIPSE, United diagnostic industry, KSA. Other chemicals used in this study were of analytical reagent grade.

Grape seed extract preparation

Grape seed were separated manually from the grape, air-dried for one week in shade at 25-30 ^{o}C and then milled into fine powder with electrical blender. Twenty grams of dry grape seed powder were soaked in 100 ml of 20% ethanol for 3-5 days. Then the mixture was filtered and the dried extract was obtained by lyophilizing [22].

Experimental design

Forty adult male mice were divided randomly into four equal groups:

In first group (non-intoxicated group), mice received standard food and water and were injected intraperitoneally with olive oil (vehicle) only. The second group was treated with GSE in which mice were given orally GSE at a dose of 200mg /kg/day for 8 weeks. In the third group (CCl4-intoxicated), mice were injected intraperitoneally with CCl4 at a dose of 0.4 ml/kg, diluted I: I in olive oil, twice a week till the end of experiment. In the fourth group (GSE+CCl4), mice were given GSE orally at a dose similar to that in the second group and injected intraperitoneally with CCl4 as aforementioned regime in the third group.

All animals were examined daily for any clinical signs and mortality rate. At end of the experiment, mice in all groups were anesthetized with chloroform and then sacrificed by decapitation.

Histopathological examination

Small tissue specimens were collected from liver tissue of mice in all the experimental groups and immediately fixed in 10% neutral buffered formalin. After proper fixation, the fixed specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Then 5 mm tissue-paraffin sections were prepared and stained with H&E stain for the microscopic examination. Masson's trichrome stain technique was also carried out using standard procedure to assess the degree of fibrosis in the liver [23].

Immunohistochemical study

Immunohistochemical determination of α-SMA was done on paraffin liver tissues sections using mouse monoclonal primary antibody against α-SMA (Sigma, Munich, Germany). Antigen localization was achieved using the avidinbiotin complex (ABC) technique [24].

Serum biochemical analysis

For evaluation of liver function, blood samples were collected from each mouse in plain tube. After blood clotting, serum was harvest by centrifugation. Then sera were stored at –20 ^{o}C for biochemical analysis. Activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as albumin and total protein values were estimated spectrophotometrically using an automated analyser.

Statistical analysis

The recorded data were analyzed using Statistical Package for the Social Sciences (SPSS) software version 20 (SPSS Inc. Chicago, USA). One way analysis of variance (one way-ANOVA) test was used to compare between means of liver function parameters in control and other groups. Values in the text were expressed as means ±standard deviation (SD) and differences with $P \leq 0.05$ were considered to be statistically significant.

RESULTS

Histopathological findings

The microscopic examination of liver of mice in the 00first (Control) and second (GSE) groups showed normal histological architecture of the liver with distinct hepatocytes and sinusoidal spaces (Fig, 1-A). In contrast, the histopathological findings of the examined liver sections of mice of the third group (CCl4-intoxicated group) were represented by marked portal fibrosis with formation of pseudolobuli that enclosed by thick fibrous septa (Fig, 1-B). Extension of fibrous tissue from portal area to neighbouring portal area as portal to portal bridging and to the central veins as portal to central bridging were more prevalent in the most of the examined liver sections. Fatty degeneration together with the presence of multiple areas of hepatic necrosis were prominent. These necrotic areas were infiltrated with mononuclear inflammatory cells particularly lymphocytes (Fig, 1-C). Moreover, diffuse hydropic degeneration of hepatocytes and bile ductal epithelium with peribiliary fibrosis and severe bile ductal hyperplasia were also seen (Fig, 1-D).

The histopathological examination of the liver of mice in the fourth group (GSE plus CCl4) revealed thin fibrous strands extended from some portal areas forming incomplete portal to portal bridging in the most of the examined liver sections. In addition, most of hepatic cells were intact and only few of them were suffered from degenerative changes in the form of hydropic and vacuolar degeneration. Moreover, deposition of collagen fibres in the portal areas mixed with mononuclear inflammatory cells and necrosis of few scattered hepatic cells were also detected (Fig.1-E&F)

Liver tissue sections which were prepared from mice in all groups and stained with Masson's trichrome stain confirmed hepatic fibrosis by greenish coloration of collagen fibres. Microscopic examination of liver sections of the first (Control) and second (GSE) groups revealed pericellular and perivascular thin layer of greenish collagen (Fig. 2-A). In contrast, the examined liver sections of mice intoxicated with CCl4 showed green staining affinity of collagen in the portal areas with formation of thick fibrotic septa between the hepatic lobules. These fibrous tissues were extended from the portal area to nearby portal area as portal to portal bridging and to the central veins as portal to central bridging (Fig. 2-B). While, in the fourth group (CCl4+ GSE), there was marked reduction in deposition of collagen which evidenced by the presence of greenish thin fibrous strands extended from portal vessels forming incomplete portal to portal bridging (Fig. 2-C).

Immunohistochemical results:

In the first (Control) and second (GSE) groups, less intensely stained α -SMA-immunopositive cells were detected only in walls of the portal and central veins ,while most of the hepatic cells remained negative (Fig. 3-A). The examined liver sections of mice in the third group (CCl4) revealed increased expression of intensely stained α -SMA-positive HSCs which were distributed in the hepatic

lobules, mainly in the portal areas and bridging fibrotic septa (Fig. 3-B). Meanwhile, the examined liver of mice in GSE plus CCl4 group, showed less expression of α-SMA than those recorded in CCL4 intoxicated group where few less intensely stained α -SMA-immunopositive cells were scattered through the hepatic lobules particularly around blood vessels, in walls of the hepatic sinusoids and in vicinity to thin fibrous strands (Fig. 3-C).

Serum biochemical results

As shown in table 1 and Graph 1, IP injection of CCl4 was followed by significant increases in mean activities of serum AST and ALT to 292.6 ± 23.93 U/L and 408.1 ± 26.58 U/L respectively comparing with those recorded values in control group where mean activities of serum AST and ALT were 37.00 ± 2.82 U/L and 40.79 ± 3.13 U/L respectively. Co-administration of GSE with CCl4 (fourth group) was associated with non-significant slight elevation in mean activities of AST and ALT to 46.89 ± 6.17 U/L and 50.32 ± 4.65 U/L respectively comparing with the recorded values of these hepatic enzymes in control non-intoxicated group. At the same time, serum AST and ALT activities in GSE with CCl4 group were significantly reduced comparing with those recorded values in CCl4 group.

In addition, IP injection of CCl4 alone resulted in significant decreases in mean levels of serum albumin and total protein to 1.37 ± 0.19 g/dl and 3.83 ± 0.19 g/dl respectively as compared to normal control group. Meanwhile in fourth group (GSE+CCl4) the mean values of serum albumin and total protein were 3.08 ± 0.12 g/dl and 5.74 ± 0.13 g/dl respectively and these values were more or less within ranges of respective values in control non-intoxicated group (3.47 ± 0.07 v and 6.11 ± 0.17 g/dl).

DISCUSSION

Hepatic fibrosis is commonly results from ongoing hepatitis and hepatic necrosis that occurs in most types of chronic liver diseases. Without treatment, liver fibrosis may lead to cirrhosis, liver failure, and liver cancer [7].

In the present study, liver fibrosis was induced in mice via IP injection of CCl4. Many previous studies demonstrated that CCl4 is a potent hepatotoxic agent caused hepatic damage through induction of membrane lipid peroxidation and generation of reactive oxygen species in tissues [25,26]. In chronic liver diseases, this oxidative stress play an important role in mechanism of liver fibrosis through activation of hepatic stellate cells and their trans differentiation into myofibroblast- like cells[27].

The examined liver of mice in intoxicated CCl4 group showed marked hepatic fibrotic lesions confirmed by Masson's trichrmone staining and represented by psudolobulation of hepatocytes with deposition of collagen in the portal areas with portal-to-portal and portal-to-central bridging. These histopathological lesions were associated with increased α -SMA-immunopositive HSCs in the hepatic lobules and in vicinity of these fibrotic lesions. These findings indicated that HSCs are accumulated in the areas of active fibrogenesis and expression of α -SMA is a characteristic indicator for development of hepatic fibrosis. These results are in a harmony with those recorded in previous experimental studies of CCl4 -induced hepatic fibrosis [28,29,30].

In addition to these hepatic fibrotic lesions, CCl4 induced hepatocellular damages in the form of diffuse hepatic degenerative changes and necrosis. These findings indicated inability of hepatocytes to protect themselves against CCl4 induced oxidative stress. In the same time, these hepatic damages were reflected on the results of serum biochemical analysis, where serum AST and ALT activities were dramatically elevated. High levels of serum AST and ALT are considered as one of serum biomarkers for hepatic damages as these hepatic enzymes are released through cell membranes of damaged hepatocytes into blood[30]. Also, IP injection of CCl4 was associated with hypoproteinemia and low albumin values that could be attributed to the disruption of protein metabolism in the liver induced by CCl4[31].

Regarding to the antifibrogenic role of grape seed extract, the results of microscopic examination of liver and biochemical analysis of serum of mice in the fourth group (GSE plus CCl4) revealed potent protective effect of GSE for hepatocytes against the hepatic damage induced by CCl4. This effect was microscopically observed by marked improvement in the liver picture where only

sporadic periportal fibrosis with incomplete portal to portal bridging and mild hepatic cellular degenerative changes were detected. This microscopic picture suggested efficacy of GSE to ameliorate CCl4-induced hepatic lesions through scavenging oxygen free radicals, inhibiting lipid peroxidation and the formation of inflammatory cytokines[32]. Similar results were also recorded in a previous study, where GSE inhibited arsenic-induced hepatic injury in rat[33].

In the present study, the recorded microscopic findings of liver in fourth group (GSE plus CCl4) were correlated with the results of serum biochemical analysis as the liver function was improved, where the recorded values of AST , ALT, albumin and total protein were within ranges of their respective parameters in the control non-intoxicated group. Additionally, immunohistochemichal study showed reducing of α–SMA expression in liver of mice intoxicated with CCl4 and co-treated with GSE where less intensely stained α -SMA-immunopositive cells were seen only around blood vessels and in walls of hepatic sinusoids.

Finally, the results of this study concluded that administration of GSE attenuated CCl4-induced hepatic fibrosis by inhibiting HSCs activation, decreasing collagen synthesis and improving hepatic regenerative capability through its powerful antioxidant and anti-inflammatory properties.

AKNOWLGDMENT

This work was kindly funded by Deanship of Scientific Research, Aljouf University, Project No. 350/35

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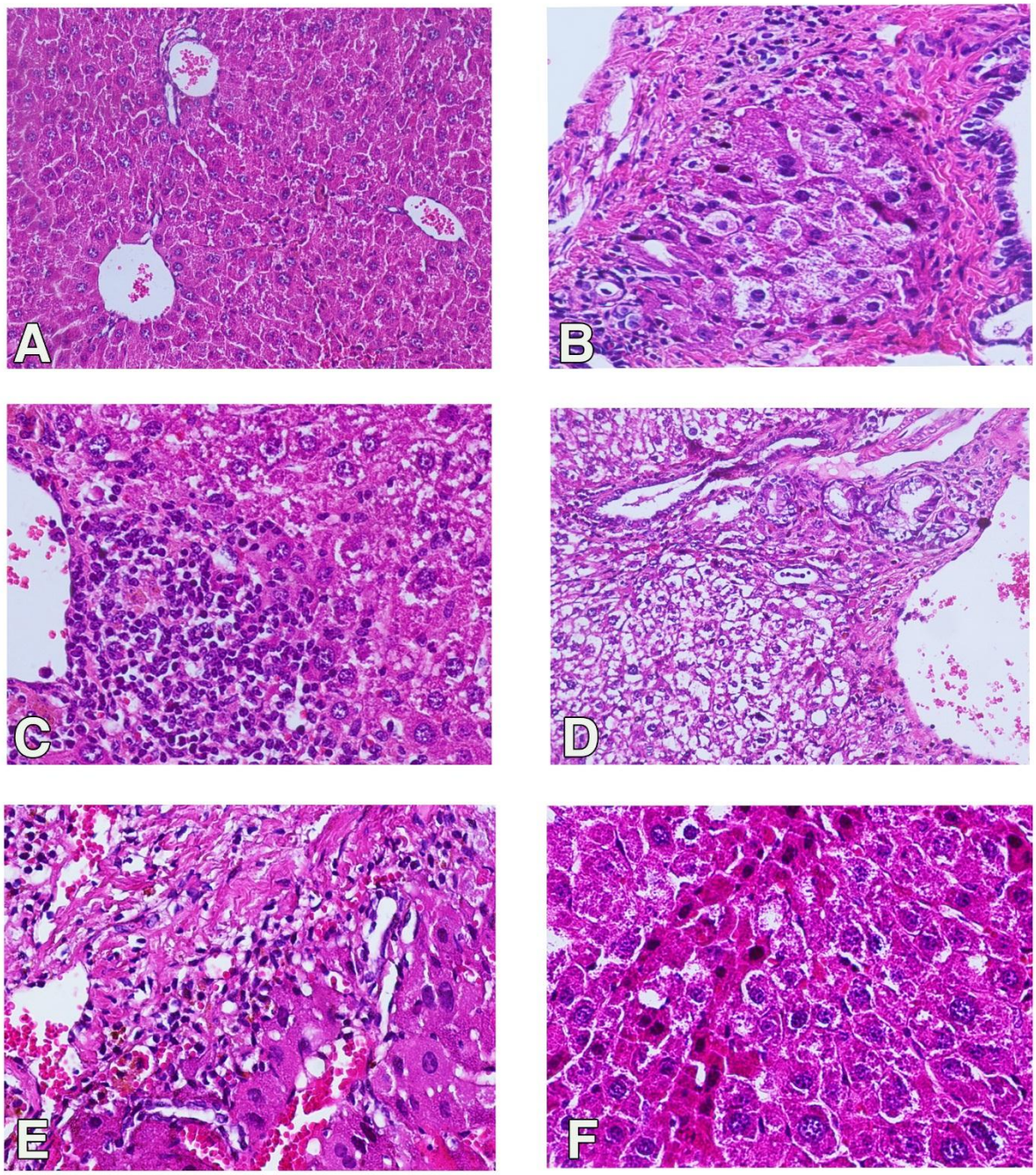


Fig.1: liver of mice in (A) control non-intoxicated group, showing normal histological architecture of the liver with distinct hepatocytes (B) CCl4 intoxicated group showing pseudolobulation of hepatocytes with thick connective tissue septa (C) mononuclear inflammatory cellular infiltration with necrotic hepatocytes (D) extensive hydropic degeneration of hepatocytes with bile ductal hyperplasia (E) GSE+CCl4 group, showing deposition of collagen fibres mixed with mononuclear inflammatory cells in the portal area (F) necrosis of few scattered hepatic cells. H &E X200.

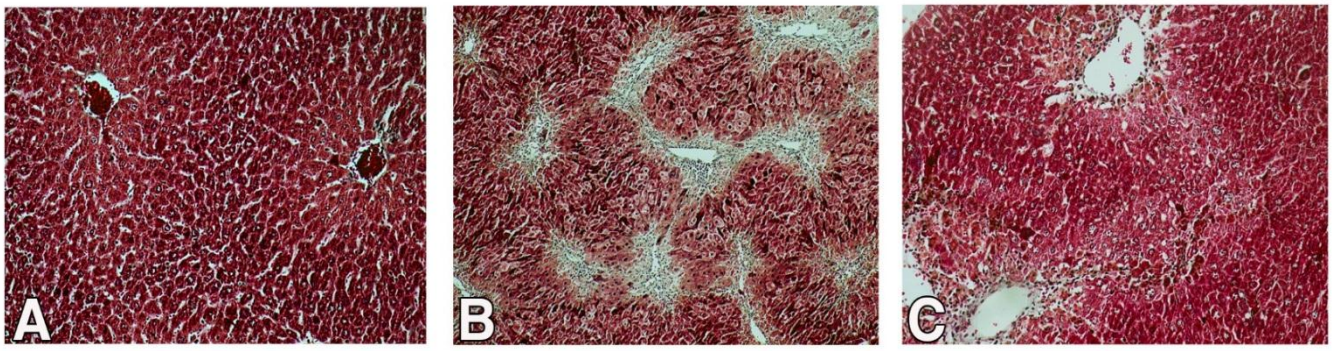


Fig.2 :liver tissue sections stained with Masson's trichrome showing (A) very thin layer of collagen around central veins in control non-intoxicated group, (B) extensive portal fibrosis with portal to portal bridging in CCl4 intoxicated group, (C) scanty periportal fibrosis with very thin incomplete portal to portal bridging in GSE+CCl4 group. X100.

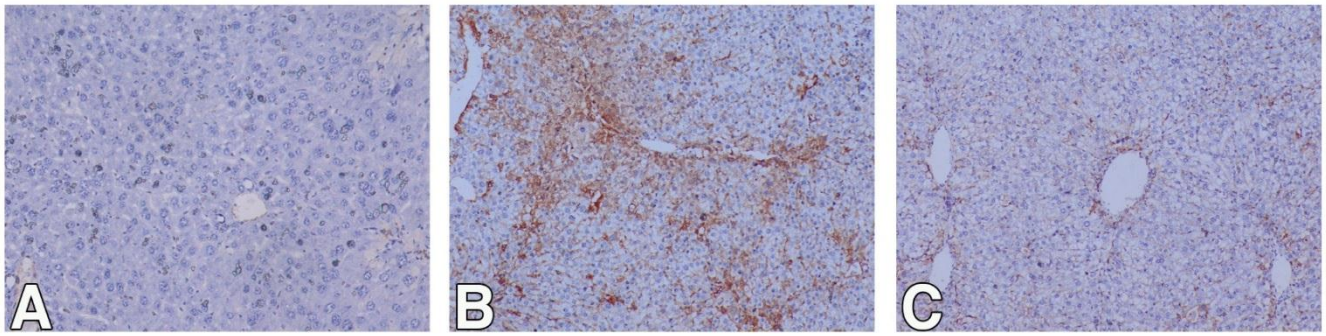
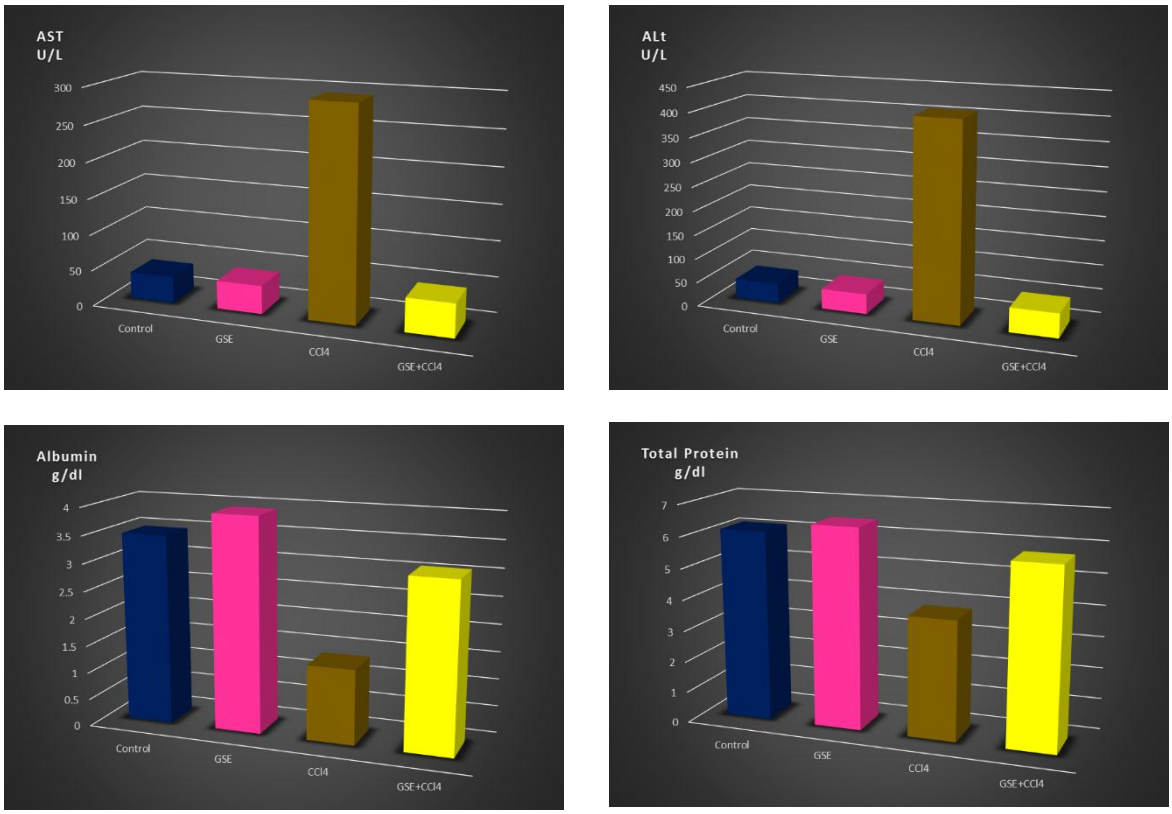


Fig. 3 : expression and distribution of α-SMA in liver of mice in (A) control non-intoxicated group showing α-SMA immunopositive cells limited to the wall of blood vessels (B) CCl4 intoxicated group showing numerous intensely stained α-SMA-immunopositive cells diffusely distributed in hepatic lobule and fibrotic bridging (C) GSE+CCl4 group showing scattered α-SMA-immunopositive cells around blood vessels , in the wall of sinusoids and in vicinity to thin fibrous strands lesions.
Immunohistochemical stain for α-SMA X100.

Table 1- Serum AST, ALT, albumin and total protein levels in control and treated groups

Group	AST U/L	ALT U/L	Albumin g/dl	Total protein g/dl
Control	37.00 ± 2.82	40.79 ± 3.13	3.47 ± 0.07	6.11 ± 0.17
GSE	39.42±2.30	41±74+2.52	3.92±0.10	6.43±0.14
CCl4	292.6 ± 23.93[a]	408.1 ± 26.58 [a]	1.37 ± 0.19 [a]	3.83 ± 0.19 [a]
GSE+CCl4	46.89 ± 6.17 [b]	50.32 ± 4.65 [b]	3.08 ± 0.12 [ab]	5.74 ± 0.13[b]

Data are expressed as Mean ± SD (n=10 mice).
a=*P ≤ 0.05 considered to be statistically significant between treated groups vs control group.*
b=*P ≤ 0.05 considered to be statistically significant between treated groups vs CCL4 group.*



Graph 1- Serum AST, ALT, albumin and total protein levels in control and treated groups.