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Hepatoprotective potential of vitamin D on liver fibrosis induced in rats by Thioacetamide

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ABSTRACT

Background: Hepatic fibrosis is a serious disease that causes around 1.5 million deaths annually due to cirrhosis and primary liver cancer. Vitamin D_3 is a sunlight hormone which promotes proliferation of cells, differentiation, apoptosis, and angiogenesis associated with hepatic parenchyma. **Aim:** Our aim was to determine potential of vitamin D to inhibit progression of hepatic fibrosis induced in albino rats. **Materials &Methods:** Thioacetamide was used to induce hepatic fibrosis in a rat model. Randomly, forty albino rats were classified into four identical groups (ten rats per group). These groups were Healthy group, fibrotic group, Prophylactic group with vitamin D and treated group with vitamin D. Biochemical and histological analysis were evaluated. By using ELISA, IL-6, IL-1B, and TNF-α were examined. **Results:** The results revealed that there was very highly significant decreased ($P<0.0001$) in ALT, ALP, AST, Total Bilirubin and Direct Bilirubin levels in groups which treated with vitamin D (G3, G4) compared with fibrotic group (G2), while there was highly significant increase in albumin ($p>0.001$) in vitamin D treated group. Liver sections from vitamin D groups showed markedly decreased hepatic lesions. We found a very highly significant effect of vitamin D on IL-6, IL-1B, and TNF-α levels**. Conclusion:** There are a potential therapeutic value and antifibrotic effects of Vitamin D on hepatic fibrosis in the thioacetamide model.

Introduction:

Nowadays, Liver fibrosis is the most important disease. More than 100 million people globally are suffering from fibrosis. concerning the prevalence of mortality [(1)]. Hepatic fibrosis is a response to chronic liver inflammation, which ultimately leads to severe accumulation of extracellular matrix and changes the healthy liver architecture [(2)]. There are several well-

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known risk factors for chronic liver fibrosis including infection of virus, drugs, autoimmune hepatitis, alcohol overuse and metabolic variations [(3)].The negligence of liver fibrosis leads to hepatic cirrhosis, hepatocellular carcinoma, and finally to liver failure [(4)]. It was long believed that fibrosis was an irreversible condition, but drugs have shown evidence of its potential to heal or even reverse [(5)]. Although, the rates of effective therapies and/or direct fibrolytics remain minimal. As a result, the need for developing novel therapies for patients with established hepatic fibrosis is increasing [(6)]. Thioacetamide is a hepatotoxicant model that causes excessive cells destruction with extremely harmful consequences on macromolecule biosynthesis. Moreover, various studies proved that TAA exposure cause liver damage, fibrosis, and cirrhosis in animal models [(7)]. Vitamin D is a pro-hormone that demands enzymatic modification of 25 and 1α-hydroxylation in the liver and kidney, respectively, leading to the production of the active vitamin D $1,25(OH)_2D_3$ [(8)]. Vitamin D has been shown in numerous studies to have nonclassical extra skeletal actions, In addition to its classic functions in bone mineralization and systemic calcium homoeostasis regulation. $1,25(OH)_2D_3$ treated Murine lung fibroblasts. additionally, $1,25(OH)_2D_3$ stimulate lung epithelial cells into myofibroblasts while restrict the trans-differentiation of TGF-β1 [(9)]. current studies on mesenchymal multipotent cells demonstrated that treatment with vitamin D caused cell cycle arrest, which led to an antiproliferative effect [(10)]. In addition, vitamin D decreased the expression of collagen and pro-fibrotic factors while increasing the rate of antifibrotic factors, such as BMP7 and MMP8 [(11)]. 22-oxacalcitriol, which is analogous to vitamin D, also prevented peritoneal sclerosis. Treatment with 22-oxacalcitriol decreased the accumulation of collagen III and myofibroblasts and inhibited the inflammatory response [(12)]. Paricalcitol (19-nor-1,25- hydroxy vitamin D_2), a synthetic analogous to vitamin D, treated mouse- obstructed kidney model significantly reduced the fibrotic lesions [(13)]. So that, the current study investigated the possibility of vitamin D hepatoprotection for the treatment and prevention of induced liver fibrosis in rats.

Materials and Methods:

Experimental animals: In the current study, all methods were approved by the ethical committee, Faculty of Pharmacy, Mania University approval code **(MPEC 230203).**This study was carried out on forty physically healthy Wistar albino male adult rats weighing 170–210 gm that was taken from the National Research Center's animal housing (Giza, Egypt). National Research Center (Giza, Egypt) approved the rats from liver-free injuries. Rats were maintained at Deraya University animal house unit (Mania, Egypt) and provided with a standard laboratory diet and tap water ad libitum. The rats were kept in a 22–25°C, 12-hour light/dark cycle air-conditioned environment. Every animal received human interest, and our study was completed with respect to the ethical guidelines for care.

Experimental design:

Randomly, Albino rats were classified into 4 experimental groups, ten rats in each group, as follows:

Group (I): Healthy group.

This group didn't receive any treatments and served as control group.

Group (II): liver fibrotic group.

Animals of this group were injected intraperitoneally with Thioacetamide (Sigma-Aldrich, USA) at a dose of 20mg/100g. body weight which dissolved in distilled water twice week for up to 8 weeks [(14)]. This group injected only by TAA and served as positive fibrotic group.

Group (III): **prophylactic group.** This group was treated Concomitant administration of Vitamin D (Sigma-

Aldrich, USA) (0.5µg/100g body weight) dissolved in distilled sesame oil [11, 15]. starting with TAA injection at a dose of 20mg/100g.body weight which dissolved in distilled water two times weekly for up to 8 weeks.

Group (IV): (Treated group)

This group was given Vitamin D (0.5µg/100g. body weight) dissolved in sesame oil two times weekly for up to 8 weeks after TAA injection period.

Serum & Tissue sample collection

By the end of the experimental period, the rats had a 12-hour fast then anesthetized by urethane (1.5 g/kg i.p.) and sacrificed. Blood specimen was assembled and centrifuged for 10 minutes at 5000 rpm to collect serum using centrifuge (Jantezki, T30, and Germany). Then Sera was kept frozen at -80°C for biochemical examination. The liver of each rat was rabidly dissected, left to dry on filter paper then weighted for calculation liver weight index. The liver was washed by cold saline and kept in 10% formalin for histopathological examination.

Biochemical Analysis

The levels of ALT, AST, ALP, Alb, Total Bilirubin and Direct Bilirubin were measured using standard procedures and commercially available colorimetric assay kits (Sigma-Aldrich).

Histopathological examination of liver tissues

Liver histopathology was determined by microscopic observation and photography. Liver samples of each animal were carefully collected at the end of the experiment. Samples were immersed in a 10% formalin solution and processed to get paraffin sections with 4 - 5 µm thickness. Hematoxylin and eosin were used to stain the sections, and a light microscope (Olympus, Japan) with a digital camera was used to observe them.

Analysis of pro inflammatory cytokines

Proinflammatory cytokines such as IL-6, IL-1β, and TNF-α were detected using ELISA kits (Elabscience, Houston, Texas, USA), based on the manufacturer's guidelines.

Statistical analysis

The statistical software package "SPSS 22.0 for Microsoft Windows were used for all statistical analysis and considered statistically significant at a two-sided $P < 0.05$. Numerical data were presented as mean ± SD.

Results

Effect of vitamin D on liver index

Firstly, we measured the body weight, liver weight and liver index of rats in each group. As shown in ***Table 1,*** the rats in the fibrotic group exhibited higher liver index and liver weight compared to the normal group. Fibrotic rats treated with vitamin D represented with obviously decreased liver index and liver weight, as shown in ***Figure 1.***

Effect of vitamin D on liver enzymes

The induction of hepatic fibrosis with TAA resulted in very highly significant increase ($p < 0.0001$) in the serum values of ALT, AST, ALP, total bilirubin, direct bilirubin, and albumin in comparison to the control group. Treatment with vitamin D highly significantly ($p < 0.0001$) restored normal ALT activity in compared to the TAA treated group. In addition, AST, ALP, direct and indirect bilirubin values were down regulated significantly after treatment with vitamin D compared to the TAA-treated group, as shown in ***Table 2*** **and** ***Figure 2.***

Effect of vitamin D on IL-6, IL-1β, and TNFα serum levels

Serum levels of the proinflammatory cytokines, IL-6, IL-1β, and TNF-α, following thioacetamide group were very highly significant elevation ($p < 0.0001$) compared with control group. However, their serum levels were markedly decreased ($p < 0.0001$) in rats treated vitamin D, whereas IL-6 serum levels showed a notable ($p < 0.0001$) highly decrease in groups treated with vitamin D, compared to fibrotic group.

Notably, TNF- α level was highly significant reduced ($p < 0.0001$) in groups treated with vitamin D, compared to fibrotic group as shown in ***Table 3 and*** "***Figure 3".***

Histological result

Histological analysis of rat liver tissue of all groups described in***" Figure 4"*** **(G I, "1", G II " 2a &2b" & GIII "3"and GIV "4 a, b").**

Group I, Control group showing normal liver tissue architecture. **Group II**, Fibrotic group was induced with TAA which is a potent hepatotoxic. The liver tissue showed predominant histopathological changes in the form of extensive disorganization of hepatocytes, hepatic cord dissolution. Varying degrees of cytoplasmic vacuolation and nuclear changes were occasionally observed e.g., nuclear pyknosis (eccentric, dark stained and small sized nucleus), fragmentation, or lysis "karyolitic". Previous changes were accompanied by significant congestion and dilation of portal veins, central veins, and blood sinusoids **(Fig. 2a)**. Widely distributed patchy necrosis and inflammatory cellular infiltration, mainly neutrophils concentrated close to the central veins and extending towards the portal area that observed in the severely affected areas **(Fig. 2b). Group III,** Prophylactic group: treated with TAA concomitant with vitamin D: This group shows improvement of the general architecture of liver tissue with low dilatation and congestion of central veins and blood sinusoids. Less inflammatory cellular infiltration and less cellular vacuolation was also seen **(Fig.3). Group IV,** Treatment group: treated with TAA followed by vitamin D**.** This group showed little improvement if compared with the previous group; clear cytoplasmic vacuolation and hepatic cord dissolution was observed. Less significant fibrosis in the central veins and peri-portal areas and occasional foci of lobular and portal inflammation completed the histological picture **(Fig 4a, a).**

Discussion

The important function of the liver is to maintain the general homeostasis of the body, in addition to the metabolism, storage, and redistribution of nutrients. Hepatic fibrosis is a common effect of most chronic liver diseases. scar tissue, distorted liver structure, In the end, it might become cirrhosis. Despite significant efforts, hepatic fibrosis still has no proven treatment [(16)]. Previously, Vitamin D (Vit. D) was linked with calcium/phosphorus balance, bone health, and growth. Within the previous few decades, Vit. D has been linked to different functions in cells, like cell proliferation, differentiation, immunomodulation, and cell death [(17)]. So, the target of several studies was the protective role of vitamin D in many diseases, such as hypertension, diabetes and cardiovascular disease[(18)]. Consequently, our aim depends on the role of vitamin D to prevent and treat liver fibrosis. TAA is the important hepatotoxicants used in rat models, because it is rapidly metabolized into highly reactive metabolites, leading to hepatic necrosis and oxidative stress [(19)]. It has been found that raised. ALT, AST, ALP, Direct bilirubin, and Total bilirubin linked to liver inflammation and damage [(20)].

The current investigation demonstrated that the parameter levels were significantly elevated in the fibrotic group but significantly decreased in treated groups with Vitamin D; these indicate vitamin D had a positive impact on liver function and decreased hepatic damage. The histopathological pictures showed varying degrees of degenerative changes of liver damage among the TAA toxified groups that designed to initiate the treatment of vitamin D. hematoxylin and eosin-stained sections showed replacement of normal hepatic tissue with extensive disorganization of

hepatocytes, hepatic cord dissolution. Varying degrees of cytoplasmic vacuolation and nuclear changes were occasionally observed around the central veins with marked dilatation of portal vein. On contrast, group III and IV that treated with vitamin D showed improvement of the general architecture of liver tissue.

IL-1b, TNF-α, and IL-6 are inflammatory biomarkers [21]. IL-1b plays an important part in the inflammation process, leading to tissue destruction. It has previously been demonstrated that IL-1 inhibits hepatocyte growth [22]. It has been found that IL-6 and TNF-α expression make great targets for liver regeneration.; TNF-α works as a proinflammatory mediator in liver apoptosis and is also linked to the cytotoxicity induced by TAA [23]. Kupffer cells produce TNF-α to respond to tissue damage, which triggers the expression of IL-6. TNF-α and IL-6 together stimulate adjacent hepatocytes, leading to the signal STAT3 (transducer and activator of transcription) activation and the production of many other proteins that are shared within the growth factor-mediated pathways. Previous research proved that Hepatoprotection and regeneration of the liver need the cytokine IL-6 after liver injury, but overexpression of IL-6 leads to liver damage [24, 25]. On the other hand, there was a very significant decrease in albumin level in TAA group compared to control and treated group with vitamin D. while, vitamin D showed highly significant decrease on cytokines biomarkers (IL-6, TNF-α, IL-1B).

Conclusion:

The study showed vitamin D as an anti-fibrotic in experimental rats. It can be used as a nutritional supplement in treating the liver and can be considered to have potential therapeutic value.

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Table 1. The Effect of Vitamin D On the Body Weight, Liver Weight, and Liver Index of Rats in Each Group. (Mean ± SD).

Variables	Group 1	Group 2	Group 3	Group 4
Body Weight (gm)	203.00 ± 2.73	169.33 ± 0.75	185.60 ± 3.4	184.87 ± 2.82
Liver Weight (gm)	5.60 ± 0.55	8.50 ± 0.54	6.00 ± 0.67	6.37 ± 0.52
Liver Index %	2.75 ± 0.27	5.00 ± 0.33 ^{a***}	3.23 ± 0.35^{b***}	3.45 ± 0 .29^{b***}

*** very highly significant at P >0.0001-significant between control **(G1)** and fibrotic group **(G2)**, (b) significant between Fibrotic group and treated group **(G3, G4)** in the same parameter.

Table 2. Serum liver function indicators at the end of the eighth week treatment with vitamin D against TAA-induced hepatic fibrosis in rats (Mean ± SD).

Variables	Group 1	Group 2	Group 3	Group 4
ALT (U/L)	25.92 ± 2.2	88.53 ± 5.09 ^{a***}	21.91 ± 2.68^{b***}	22.95 ± 5.38 ^{b***}
AST (U/L)	41.2 ± 1.6	122.66 ± 2.8^{a***}	51.6 ± 2.41 ^{b***}	54.5 ± 1.4 ^{b***}
ALB (g/dL)	3.88 ± 0.25	2.63 ± 0.09 ^{a***}	3.77 ± 0.22 ^{b***}	4.08 ± 0.11 ^{b***}
Total BILI (mg/dl)	0.90 ± 0.04	1.90 ± 0.05 ^{a***}	1.02 ± 0.07 ^{b***}	1.15 ± 0.19 ^{b***}
Direct BILI (mg/dl)	0.16 ± 0.019	0.72 ± 0.04 ^{a***}	0.21 ± 0.015 ^{b***}	0.24 ± 0.03 ^{b***}
ALP(U/L)	111.01 ± 7.8	405.66 ± 8.3 ^{a***}	117.8 ± 4.3 ^{b***}	143.63 ± 4.56 ^{b***}

*** very highly significant at P >0.0001-significant between control **(G1)** and fibrotic group **(G2)**, (b) significant between Fibrotic group and treated group **(G3, G4)** in the same parameter.

Table 3. Serum cytokines biomarkers at the end of the eighth week treatment with vitamin D against TAA-induced hepatic fibrosis in rats (Mean ± SD).

	Group 1	Group 2	Group 3	Group 4
IL-6 (pg/ml)	8.2 ± 0.84	37.5 ± 3.5^{a***}	10.9 ± 1.8^{b***}	13.24 ± 3.41 ^{b***}
TNF-α pg/ml)	40.2 ± 0.81	91.2 ± 3.5 ^{a***}	42.3 ± 3.9 ^{b***}	42.4 ± 3.5
IL-1B pg/ml)	18.4 ± 3.1	74.8 ± 4.3 ^{a***}	20.01 ± 2.9 ^{b***}	23.1 ± 3.6 ^{b***}

*** very highly significant at P >0.0001

(a) significant between control **(G1)** and fibrotic group **(G2)**, (b) significant between Fibrotic group and treated group **(G3, G4)** in the same parameter.

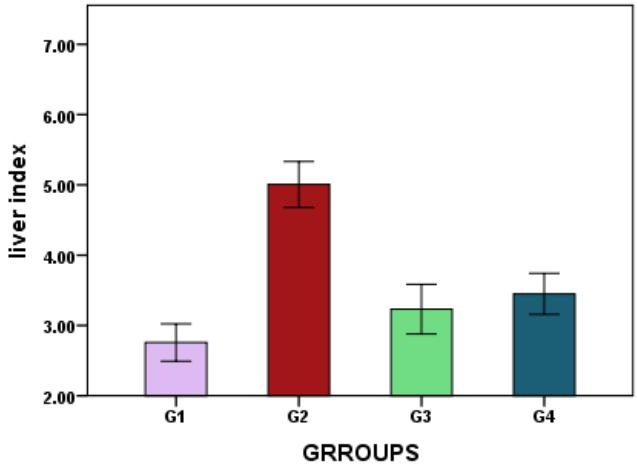
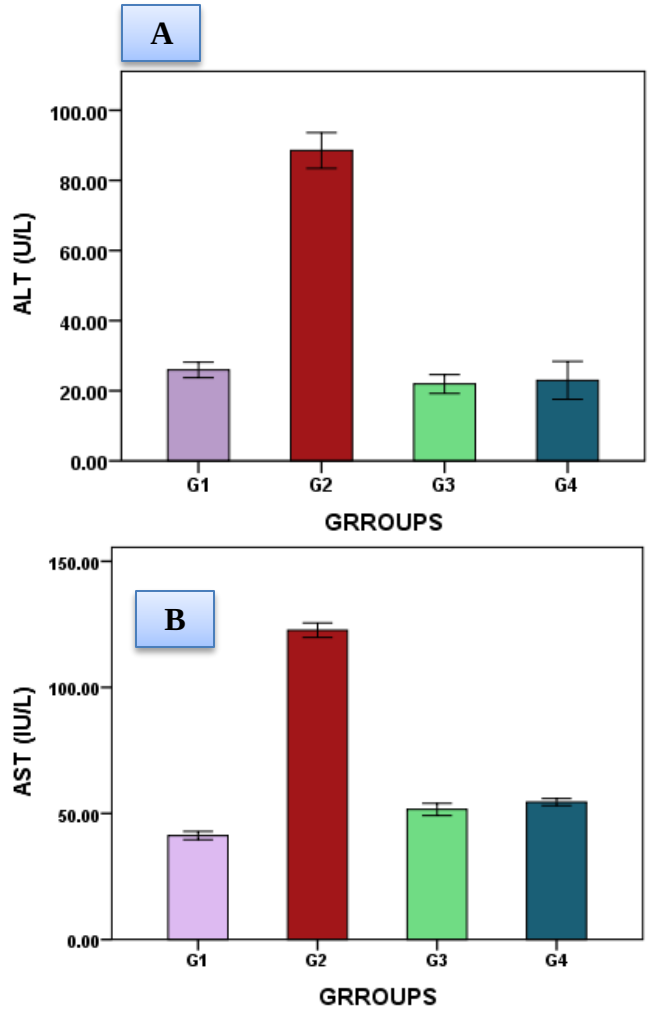
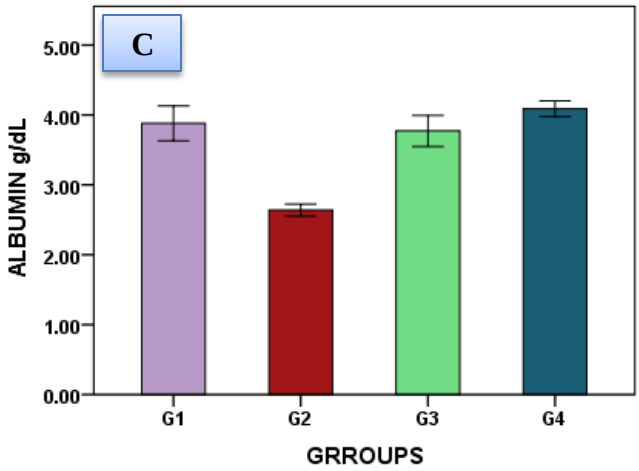


Figure 1. Liver Index. liver indices of TAA- rats (G2) was highly significant increased as compared to control rats (G1). Treatment with vitamin d (G3, G4) attenuated amplification of liver indices.

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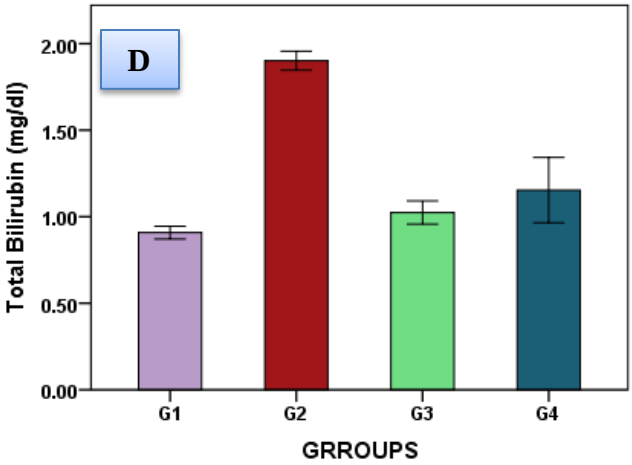
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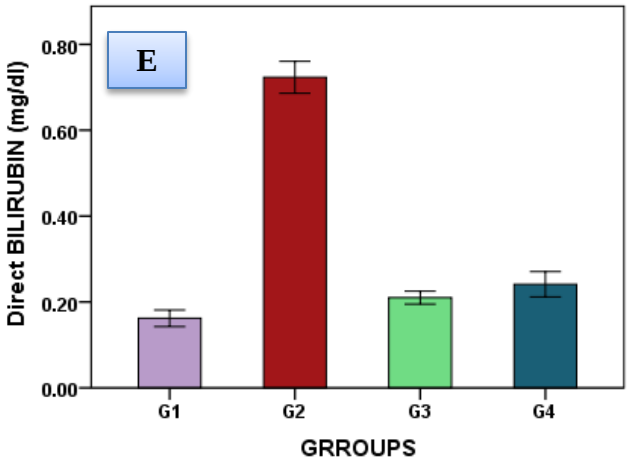
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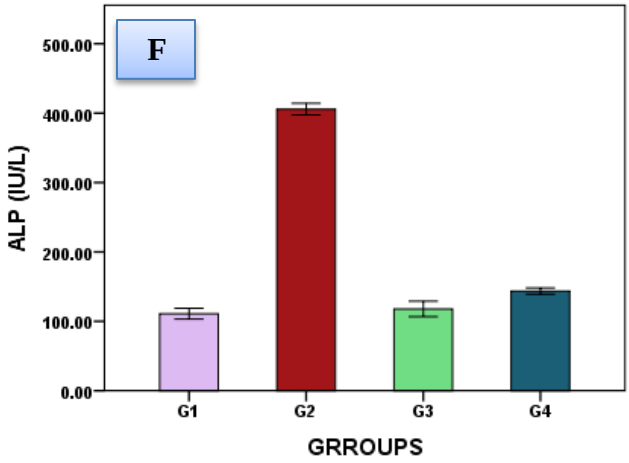

***Figure 2*. Serum levels of ALT (A), AST (B), Albumin (c). Total Bilirubin (d), Direct Bilirubin (E) and ALP (F). Bars represent mean ± SD**

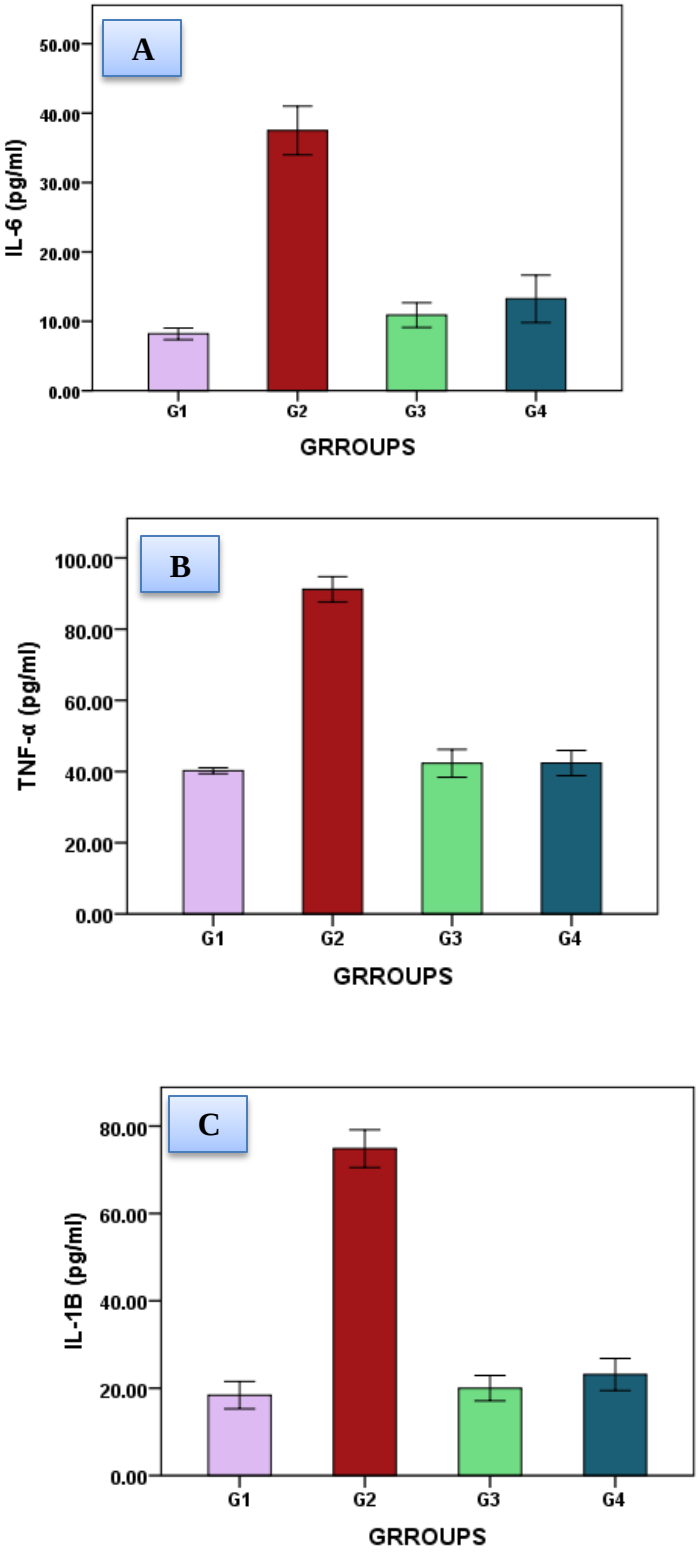

***Figure 3*. Serum levels of IL-6 (A), TNFα (B), and IL-1β (C) Bars represent mean ± SD.**

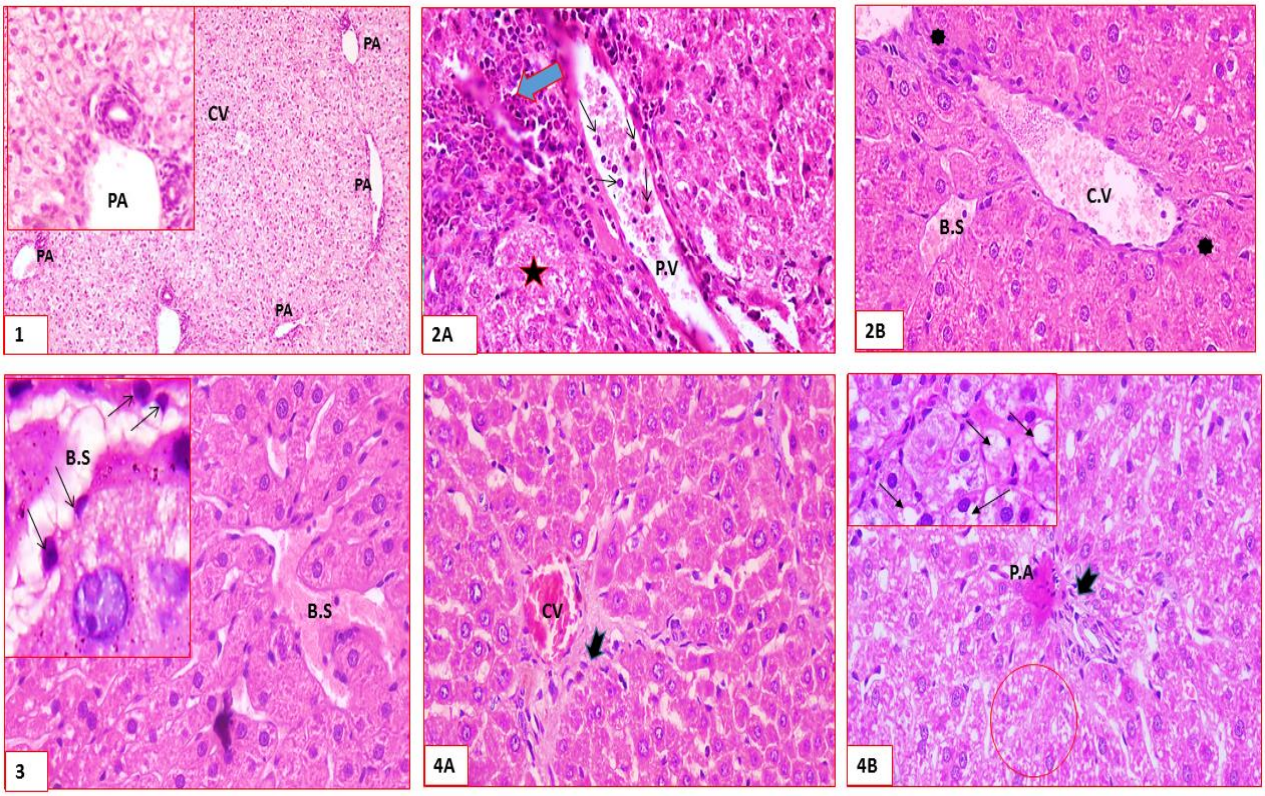

***Figure 4*: Photomicrographs of rat liver tissue; G I, "1", G II " 2a &2b" & G III "3"and G IV "4 a, b" **showing**:

1- Normal liver architecture showing hexagonal classic hepatic lobules with central v (CV) surrounded by portal areas (PA) at each corner. Liver cells arranged in cords and blood sinusoids in-between rows of hepatocytes (arrows).

2A- Extensive disorganization of hepatocytes, patchy necrosis (star) and inflammation mainly around the portal areas (arrow). Notice dilated portal vein filled with inflammatory mononuclear cells mainly neutrophils (arrows).

2B- Showing congestion of central vein (CV) and blood sinusoids (BS) and dissolution of hepatic cords (asterisks).

3- Showing less dilatation and congestion of central veins and blood sinusoids, less inflammatory cellular infiltration and less vacuolation. The inset showing higher magnification of a blood sinusoid with prominent von kupffer cells incorporated within its endothelial lining (arrows).

4A & 4B - Showing less significant fibrosis (tailed arrows) around the central veins (CV) and