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Original Article

Efficacy of interleukin-6 in the induction of liver cell proliferation after hemi-hepatectomy: histopathologic and immunohistochemical study

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Abstract: Introduction: The function of Interleukin-6 (IL-6) in the regenerative process is not fully understood. The aim was to show the IL-6 role in hepatocyte regeneration by identifying the proliferative rate of hepatocytes following partial hepatectomy. Material and methods: Eighty male adult Sprague-Dawley rats were categorized into two equivalent groups (n = 40 rats); non-treated, and treated group with IL-6 of 35 μg/100 gm body weight according to lethality study for a four-day observation. Both groups were subjected to 70% hepatic resection. Liver specimens were taken for histo/immunohistochemical studies. Five measures were investigated histopathologically; binucleation, mitoses, thickening of the hepatic plate, ductular reaction, and presence of inflammatory cells. Ki-67 labeling index was evaluated using mouse anti-Ki-67 antibody. Results: In non-treated group; binucleation and multinucleation were noted in 12 cases (30%), bizarre cells with abnormal mitoses 16 cases (42%), and thickening of liver cell plate 18 cases (45%), in contrast to 32 (80%), 30 (75%) and 28 (70%), in treated group. Patches of inflammatory infiltrate were more marked in the treated group. Ki-67 labeling index was higher in the treated group (*p*-value 0.00001). The degree of Ki-67 reactivity in the treated group was: negative 6 (15%), weak 6 (15%), moderate 16 (40%) and strong 12 (30%) compared with 18 (45%), 13 (32.5%), 6 (15%) and strong 3 (7.5%) in non-treated group. Conclusion: IL-6 is valuable in the induction of liver cell regeneration. Correlation with biochemical assay and flow cytometric studies is recommended.

Keywords: IL-6, hepatocyte, hepatectomy, Ki-67, regeneration

Introduction

The role of IL-6 in hepatocytic regeneration is mainly related to the elaboration of acute phase reactants. IL-6 is synthesized at a site of hepatocyte injury in the early inflammatory stage, followed by the rapid synthesis of acute-phase reactants such as haptoglobin, α1-antichymotrypsin, C-reactive protein, and fibrinogen [1].

Alternatively, IL-6 promotes hepatocyte stimulating factor which results in a decrease in the production of albumin, fibronectin, and transferrin. IL-6 is concerned with the iron and zinc hemostasis through controlling their absorptions. Concerning serum level of iron, IL-6 promotes the synthesis of hepcidin, which obstructs the ferroportin 1 action that acts as a carrier on GIT, and hence, decreases the level of serum iron [2]. This mechanism explains the presence of low serum iron and anemia linked with chronic inflammatory conditions. Additionally, IL-6 also improves Zinc importer 14 (ZIP14) expression in the hepatocytes and so produces hypozincemia, usually associated with inflammatory lesions [3]. IL-6 is associated with increased platelets in the peripheral blood owing to its induction of maturation of megakaryocytes [4].

Furthermore, IL-6 promotes the connection of innate with the acquired immune response by induction of naïve CD4+ T cell differentiation. IL-6 in addition to transforming growth factor (TGF)-β, is necessary for Th17 differentiation from naïve CD4+ T cells [5]. IL-6 also induces T- helper-cell differentiation and production of IL-21 [6], which regulates immunoglobulin (Ig) synthesis and IgG4 especially. Besides its role upon CD4+ cells, it stimulates the production of

cytotoxic T cells from CD8+ T cells [7]. IL-6 also has a role in the activation of B cells into plasma cells, with a marked increase in antibody production.

The IL-6 role in liver cell regeneration was first identified by Higgins and Anderson (1931) [8], who noticed a complete regeneration of the rat liver after semi-hepatectomy using surgical removal. Higgins and Anderson's study was considered as the starting point for upcoming research in hepatic regeneration. On the other hand, encouraging results were obtained from rats while investigating the activated dependent IL-6 genes after two-thirds of liver mass hepatectomy in which the elevation of TNF-α was followed by an elevation of IL-6 serum levels [9]. These events occur in the early phase before the synthesis of DNA in hepatocytes 24 hours after hepatectomy. Consequently, activation of the transcription factors occurs, especially nuclear factor-interleukin 6 (NFIL-6), leading to enhancement of their aligned genes transcription [10-13]. These factors were found to be involved in the generation of the G0/G1 phase of liver cells after hepatectomy [14].

The current study explores the role of IL-6 in liver cell regeneration, and the proliferative rate of hepatocyte regeneration following surgical partial hepatectomy using predetermined dose IL-6 injection, and compares the rate of cellular proliferation with that in rats not receiving IL-6 injection.

Material and method

The experiment was done following the Guide for the Care and Use of Laboratory Animals produced by National Institute of health, USA The rats were applied. The experiment was carried out in agreement with the ethical standards [15].

Experimental design

Eighty adult-male Sprague-Dawley rats weighted about 200-230 g were obtained from the animal residence for scientific studies, King Abdulaziz University, (Jeddah, Kingdom of Saudi Arabia). The animals were hosted in a pathogen-free environment in the animal house of the Albaha faculty of Medicine, Kingdom of Saudi Arabia, in a 12-hour light-dark cycle and meals/water and libitum are available.

Calculation of the Interleukin 6 lethality dose on rats

The male rats were randomly sub-grouped into numerous groups of 5 rats each. Treatment with different doses of 20-80 μg/100 g of IL-6 occurred for all. The number of surviving rats was counted daily for 30 days.

Following the estimation of the lethality dose, eighty male rats were grouped into two equal groups (n = 40 rats). Group 1: control group; not treated and group 2 that was subjected to the treatment with 35 μg/100 gm body weight according the lethality study. The treated group was injected intravenously daily for four days. Towards the end of the treatment period, the liver was removed, weighted and bisected longitudinally for histopathologic and immunohistochemistry studies.

Surgical procedures

Liver resection was done by using midline laparotomy after induction of general anesthesia. Buprenorphine (0.05 mg/kg), Acepromazine intramuscular of 0.8 mg/kg, and 40 mg/kg ketamine were used to start general anesthesia. The surgical resection steps done according of Higgins and Anderson steps [8].

After the midline laparotomy, liver mobilisation and exposure were achieved then the lobes are resected after ligation of the lobes origin by using a 3-0 silk tied suture. Over the superior border of the lobes, the sutures ends were approximated and closely tied at its root. Dissecting scissors was used distal to the ligated sutures to resect the lobes. Abdominal wall closure was done using a 3-0 Polyglactin suture. Finally, skin closure was done by using polyamide suture.

Histopathologic and immunohistochemical studies

Three days after the closure of the skin, the sacrifice of rats of the two groups (treated and untreated) occurred and the residual hepatic tissue was detached. Liver specimens were taken for histopathologic and immunohistochemical studies.

The liver specimens were fixed in ten percent formaldehyde solution, treated in sequential concentrations of ethanol and xylene, embedded in paraffin, and cut into 4-μm thick sec-

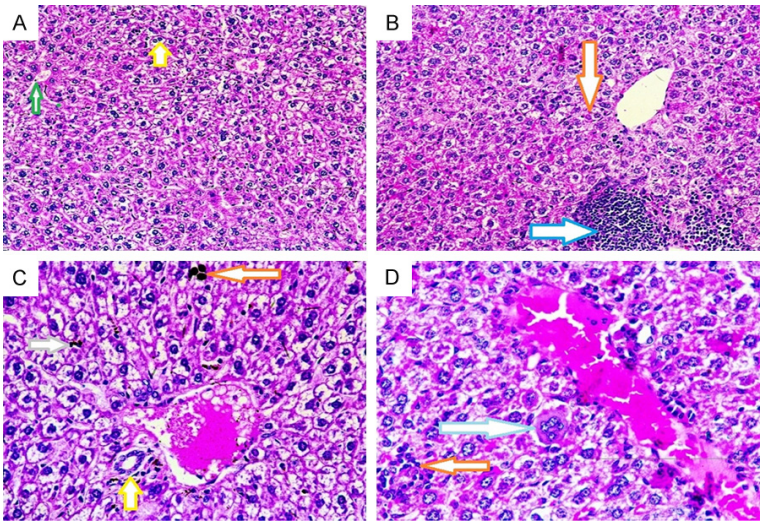


Figure 1. A. A case of control group showing thickening of liver cell plates as demonstrated by heavy cellularity and narrowing of sinusoidal lumen, in addition to the presence of binucleation (yellow arrow), sparse inflammatory cells, and ductular proliferation (green arrow) (H&E, ×100). B. A case of treated group showing distorted double cell plate (orange arrow) in addition to patchy inflammatory cell infiltrates (blue arrow) (H&E, ×200). C. A case of treated group showing inflammatory cell infiltrate (orange arrow) and ductular proliferation (yellow arrow) (H&E, ×400). D. A case of treated group showing bizarre cells with multinucleation, (light blue arrow) and inflammatory cells (orange arrow). Liver cells showing cellular and nuclear pleomorphism (H&E, ×400).

tions and stained with hematoxylin and eosin. Two sections were prepared for each liver specimen. Five factors were investigated in the histopathologic examination: binucleation of hepatocytes, mitoses, thickening of the hepatic plate, ductular reaction, and the presence of inflammatory cells. A scoring system out of five was applied for these measures.

Immunohistochemical (IHC) analyses were carried out on slides coated with poly-L-lysine. IHC for Ki-67 was carried out using mouse Anti-Ki-67 antibody (ab136152) antibody (a concentration of 100 µg at 1 mg/ml, 1:50 diluted, Cambridge, UK). The staining procedure was automated and consisted of incubation for 45 minutes with the primary antibody, then washing by a brief buffer followed by incubation with biotinylated anti-mouse IgG/IgM manufactured by Santa Cruz Biotechnology (Santa Cruz, California, USA) for thirty minutes. The slides were subsequently incubated with avidin/biotin (Biotech) for 30 minutes and reacted with diaminobenzidine (DAB) and hydrogen peroxide (H_2O_2). Assessment of reactivity was done with brown nuclear staining of regenerating liver cells considered as positive. The staining intensity and the percentage of positive cells were calculated to assess the ki-67 labeling index, in which five fields per specimen were haphazardly selected. Positive cells were calculated in high-power fields (×400) and the mean number of positive cells per limited surface area was counted.

For statistics, the reactivity was considered from both domains; the proportion of cells stained Ki-67 positive was rated 0 = negative, weak = 1, moderate = 2 and strong = 3 and the degree of intensity (which denotes the concentration of the stain in positive cells) was graded from (0) for negative staining and +, ++, and +++ for weak, moderate and strong intensity. The percentages of positive cells were calculated as follows: 1+ (weak) = less than 10%, 2+ (moderate) = 11 to 50% and 3+ (strong) = more than 50% tumor cells stained positive [16]. Positive endothelial cells were considered an internal control.

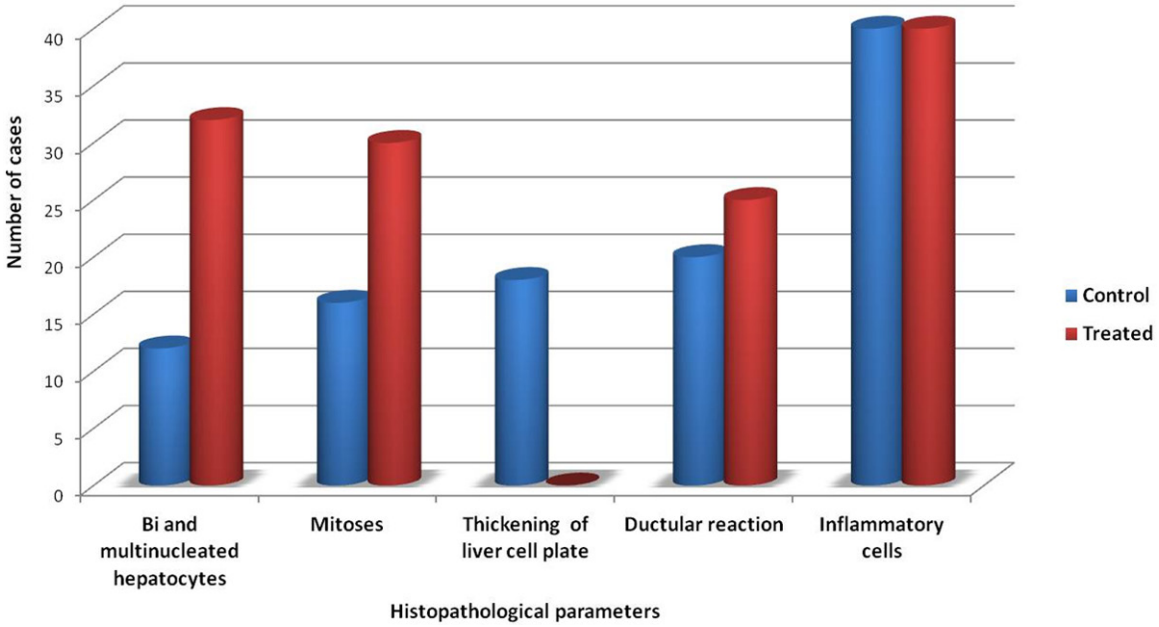
Mann-Whitney test was utilized for comparison of the histopathologic findings of the two groups, in addition to Ki-67 expression with both groups. A two-sided p-value of ≤ 0.05 was used. Statistical Package for Social Science (SPSS) version 18 was used in the analysis.

Results

Histopathologic evaluation of regenerated hepatocytes was based on the triad of hepatic regeneration which consists of the predominance of binucleated hepatocytes, increased number of mitoses, and thickening of liver cell plates (**Figure 1**). Histopathologic examination revealed the presence of this triad in the control group as follows: binucleation (**Figure 1A**) and multinucleation (**Figure 1D**) (12; 30%), bizarre cells with abnormal mitoses/nuclear chromatin condensation with prominent nucleolus in 16 (42%), and thickening of liver cell plate in 18 (45%), in contrast to 32 (80%), 30; (75%) and 28 (70%) for the treated group

Table 1. Summary of histopathologic findings in control and treated groups

Histopathological findings		Control group	Treated group	Mann-Whitney test
		Number & Percent		
1	Bi- and multinucleated hepatocytes	12; 30%	32; 80%	*P = 0.0106*
2	Mitoses	16; 42%	30; 75%	
3	Thickening of liver cell plate	18; 45%	28; 70%	
4	Ductular reaction	20; 50%	25; 62%	
5	Inflammatory cells	40; 100%	40; 100%	



Graph 1. Histopathologic findings in both control and treated groups.

(**Figure 1D**), respectively. The presence of scattered inflammatory cells was seen in both control (**Figure 1A**) and treated cases (**Figure 1C**), but patches of inflammatory infiltrate were more marked in the treated group (**Figure 1B**). This significant cell proliferation gives rise to the thickening of the liver cell plate which showed the arrangement of more than two hepatocytes leading to a narrowing of the sinusoidal lumen. Ductular reaction with proliferating cholangiocytes and oval cells were seen in more than 50% and 62% of control and treated groups, respectively. These data are summarized in **Table 1** and expressed in **Graph 1**.

Results of immunohistochemical staining

Ki-67 reactivity was limited to the nucleolus and nucleus, principally the nuclear membrane. In non-treated liver sections, there were several ki-67 positive cells with mean labeling index 8±.641723 versus 18±2.815476 in the treated group. Some hepatocytes showed both nuclear and cytoplasmic staining (**Figures 2**, **3**).

Detailed nuclear ki-67 expression occurred in 85% (**Figure 2** and **Table 2**) (**Graph 2**). The degree of reactivity in the treated group was graded as negative (6,15%), weak (6, 15%), moderate (16, 40%), and strong (12, 30%) compared with negative (18, 45%), weak (13, 32.5%), moderate (6, 15%), and strong (3, 7.5%) in the control group (**Figures 2**, **3**). The intensity of staining in the positive cases in the treated group, (total = 34/40) was as follows: (+ = 8/34; 23.5%), (++ = 12/34; 35.2%), and (+++ = 14/34; 41.1%) compared with (+ = 9/22; 40.9%), (++ = 7/22; 31.8%), and (+++ = 6/22; 27.2%) in the control group. The distribution of the positive Ki-67 reactivity was more

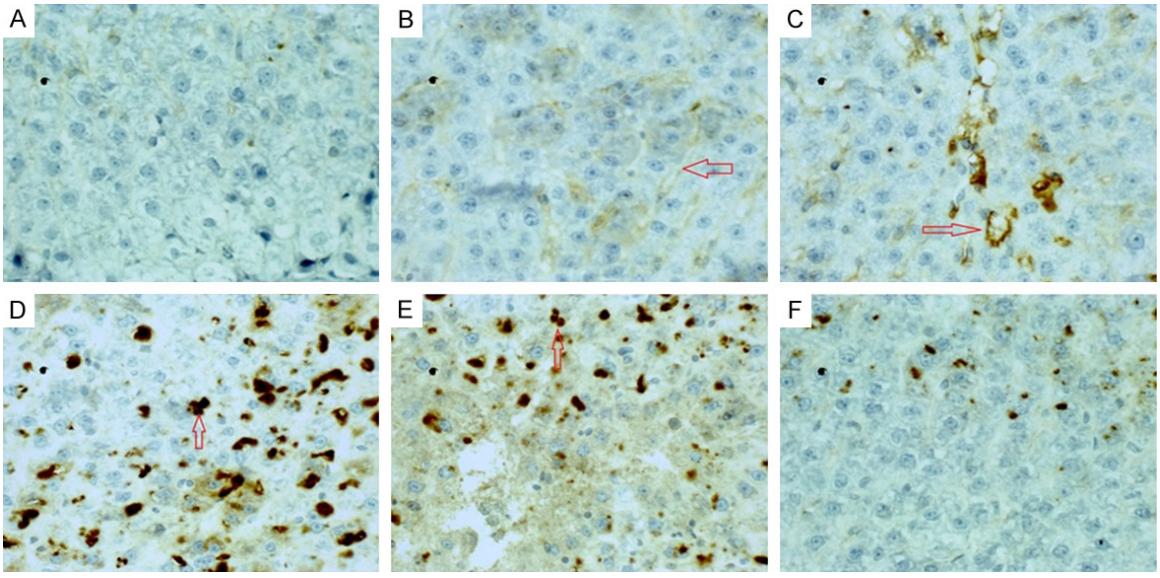


Figure 2. Sections for estimation of Ki-67 labeling index. Paraffin sections of rat liver obtained from different control cases showing different grades of Ki-67 immunoreactivity: (A) Negative, (B) Sinusoidal (arrow), (C) Ductular, (D) Moderate, mitoses (arrow), (E) Strong staining for lymphocytes (arrow), and (F) Mild and focal scattered reactivity. (D-F) Positive nuclei exhibit strong intensity for Ki-67. These different sections were taken to estimate the Ki-67 labeling index. (DAB, IHC of Ki-67 ×400).

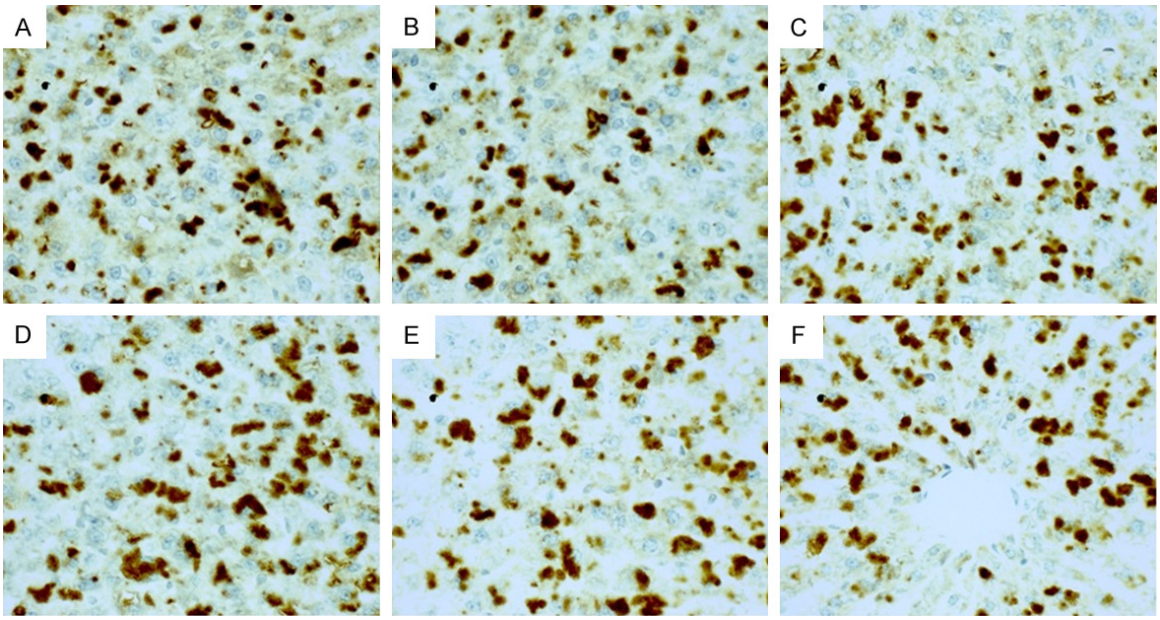


Figure 3. Sections for estimation of Ki-67 labeling index. Paraffin sections of rat liver obtained from different treated cases with IL-6 showing Ki-67 immunoreactivity. These different sections were taken to estimate the Ki-67 labeling index. All the positive nuclei exhibit strong intensity for Ki-67 immunostaining. (DAB, IHC of Ki-67 ×400).

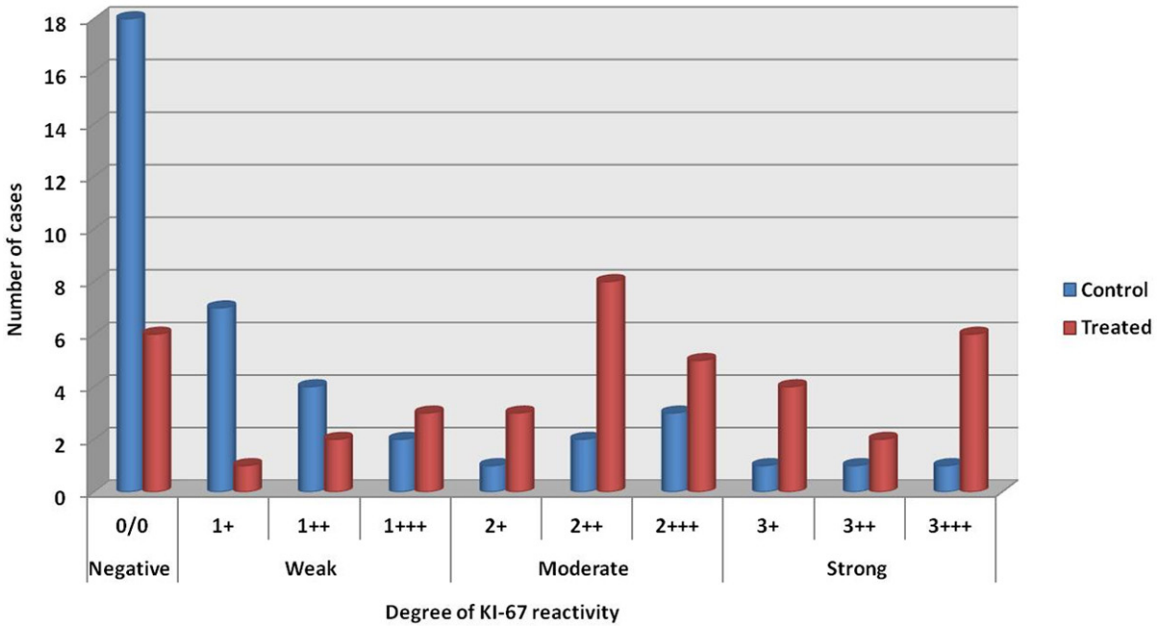
marked in the centrilobular than the peripheral zone which was opposite to that of the control group where it was more marked in the peripheral than the centrilobular zone (**Figures 4**, **5**). A differential reactivity score, which includes the percentage and intensity of both groups, is summarized in **Table 3**.

Discussion

IL-6 guards liver cells against ischemic change and hence, promotes hepatocyte reperfusion and proliferation [17]. However, its role in the regeneration process, especially after partial hepatectomy, is not well documented despite

Table 2. Differential Ki-67 reactivity score, including the percentage of positive cells and the degree of intensity in control and treated groups

Degree & percentage of reactivity (0-6)		Number & Percent		Mann-Whitney test
		Control	Treated	
Negative	0/0	18 (15%)	6 (15%)	*P* value = 0.00001
Weak	1+	7 (2.5%)	1 (2.5%)	
	1++	4 (5%)	2 (5%)	
	1+++	2 (7.5%)	3 (7.5%)	
Moderate	2+	1 (7.5%)	3 (7.5%)	
	2++	2 (20%)	8 (20%)	
	2+++	3 (1.25%)	5 (1.25%)	
Strong	3+	1 (10%	4 (10%)	
	3++	1 (5%)	2 (5%)	
	3+++	1 (15%)	6 (15%)	



Graph 2. Degree of Ki-67 reactivity in the control and treated group in the present study.

reports that have recognized the central role of IL-6 in the regenerating liver cell after resection or injury [18-21].

In the present study, histopathologic examination revealed that signs of liver cell regeneration (binucleation, mitoses, thickening of liver cell plate with narrowing of sinusoidal space, and inflammatory cell infiltrates and ductular reaction) were significantly more marked in the treated group than the control group.

These results are following studies of Dancygier [22], Fausto et al [23], and Castaing et al [24] who stated that signs of liver cell regeneration following injury include increased rate of cellular proliferation and mitoses resulting in the thickening of liver cell plates with prevalent double-cell plates, which compress the sinusoids and obliterate them.

As established herein, in the treated group, exogenous IL-6 may hasten the process of regeneration and consequently, restoring of liver cell mass, compatible with the observations stating that the IL-6 can pose therapeutic benefits in the prevention of organ ischemic injury during preservation before transplantation [25-28]. It was suggested that IL-6 may

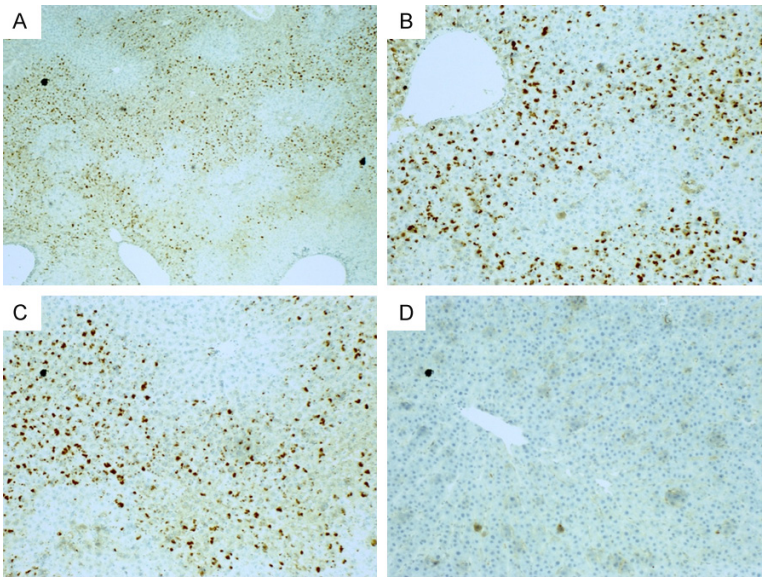


Figure 4. A case of the control group, (A-C) is showing that the hepatocytes demonstrate moderate expression for the Ki-67 antibody, score 4/6, DAB (×100). The pattern of reactivity is more in the peripheral than the centrilobular zone. (D) Negative Ki-67 expression (IHC, DAB, ×200).

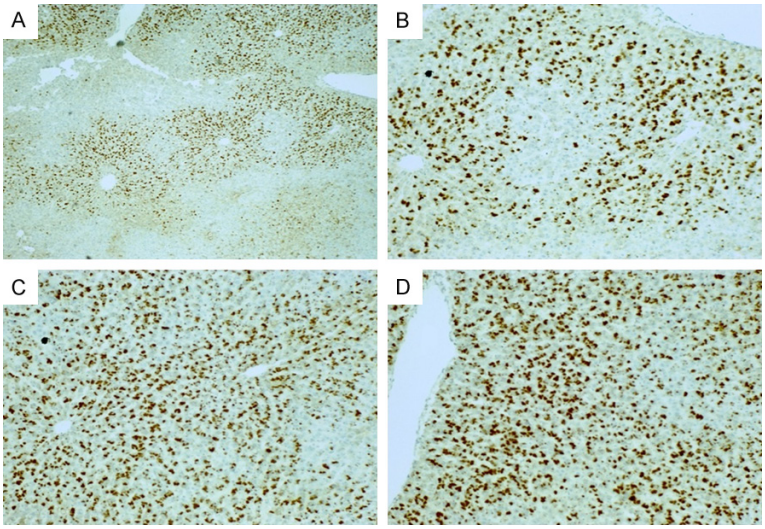


Figure 5. A case of the treated group, (A) Different grades of immunoreactivity for Ki-67. Most of the hepatocytes exhibit strong nuclear Ki-67 staining, more marked in the centrilobular zone than in the peripheral zone. Mean reactivity score ranged from 4/6 in (A) (×100) and (B) (×200), to 5 in (C) and 6 in (D) (×200), (DAB, IHC). Graph 1. Histopathologic findings in both control and treated groups. Graph 2. Degree of Ki-67 reactivity in the control and treated group in the present study.

modify the resistance of glucose in vivo and may contribute to resistance of insulin seen in diabetes [29, 30]. Besides, a short-term IL-6 administration prevents apoptosis and hence, promotes and hastens the restoration of liver cell mass after injury or hepatectomy and validates the observations of Kovalovich et al [31] who stated that IL-6 is linked with the reduced activation of the apoptotic pathway in the mitochondria opposed to increased activation and expression of the anti-apoptotic family members including Bcl-xl and Bcl2 [32]. These data confirm the critical role of IL-6 as a promoting factor in liver regeneration [33, 34].

Also, the results obtained agree with Zimmers et al [34] who stated that mice with IL-6 deficiency showed delayed liver cell regeneration. Also, they revealed that IL-6 administration in nude mice resulted in marked liver cell hyperplasia and hepatomegaly in the absence of liver injury, which reflected in an increased liver to body mass ratio.

The results obtained are contrary to reports that stated that IL-6 inhibited hepatocyte regeneration and delayed the repair and regenerative process [35]. Jin et al [36] examined long-term IL-6 administration and its effect on the recovery from hepatic resection and found an impaired repair and restoration of liver cell mass. They concluded prolonged exposure to IL-6 in vivo induces apoptosis and is considered an anti-regenerative factor.

Ki-67 as a proliferative marker was more expressed in the treated group as indicated by the high labeling index of the treated group compared with that of the non-treated group with highly significant p-value obtained. This result is in agreement with the study of Gerlach et al [37] who revealed the efficacy of the MIB-5 antibody in monitoring the

Table 3. Scoring of the histopathologic findings in control and treated groups

Histopathologic score out of five	Control group	Treated group	Mann-Whitney test
	Number & Percent		
0/5	4; 10%	2; 5%	*P = 0.00528*
1/5	13; 32.5%	6; 15%	
2/5	12; 30%	9; 22.5%	
3/5	9; 22.5%	15; 37.5%	
4/5	1; 2.5%	3; 7.5%	
5/5	1; 2.5%	5; 12.5%	

proliferation of rat liver, and besides, the pattern of the mRNA and the protein expressions suggests that the Ki-67 protein is more abundant in the process of DNA synthesis. Also, it coincides with the study of Andersen et al [38] who found that hepatocyte proliferation indicated by ki-67 reactivity was high in 1-3 postoperative days. Haldrup et al [39] studied the liver regenerative capacity following partial hepatectomy in rats with non-alcoholic steatohepatitis and found that ki-67 was higher in high fat and cholesterol diet animals and concluded that non-alcoholic steatohepatitis rats had a conserved liver regeneration capacity following hepatectomy compared to standard diet rats. Aguiar et al [40] found a positive correlation between liver cell regeneration and the elevation of portal pressure.

In the present work, the IL-6 induced proliferation in centrilobular and mid-zone compared to peripheral zone may be attributed to presence of heterogeneity in the lobular distribution of peroxisomes as stated by Gorgas et al [41]. They found some ultrastructural differences between cells of centrilobular zone versus the peripheral zone, one of these was the shape and size of peroxisomes. These were largest and most frequent in the centrilobular region, demonstrating that these cells are most responsive to peroxisome proliferation and hence increase its affinity for staining.

Conclusion

IL-6 is valuable in the induction of liver cell regeneration as evidenced by marked histopathologic reparative changes and high reactivity with the proliferation marker Ki-67. These results need to be confirmed with other measures such as liver enzymes and DNA estimation by flow cytometry.

Disclosure of conflict of interest

None.

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