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BIOCHEMICAL, IMMUNOLOGICAL AND HISTOPATHOLOGICAL CHANGES OF RAT LIVER INDUCED BY CARBONYL IRON

By

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ABSTRACT

Abstract : Iron is a necessary element for all living cells. Its deficiency with or without anemia is the main cause of nutritional deficiency for human beings. In other words, there are many forms of diseases associated with excess iron storage. The clinically most important of which is hereditary hemochromatosis (HH). The present study was undertaken to investigate the correlation between biochemical, histopathological and immunological changes in the parameters in liver injury models induced by carbonyl iron. 42 Sprague - Dawley rats were used in this study, 12 rats as control and 30 rats were treated with carbonyl iron at a dose of 3% of the chew diet for 24 hrs, 72 hrs, 1 week, 4weeks, 8 weeks and 12 weeks. The activity of serum transaminases (ALT and AST), antioxidant enzymes (SOD, GSH and CAT), and blood platelets count and serum auto antibodies (ANA, ASMA and AMA or LKM) were determined. Liver samples of the control and treated animals at different times were stained with hematoxlene and eosin, Masson's trichrome and Perl's Prussian blue stains. Liver fibrosis was graded biochemically by liver fibrosis markers (serum collagenase, hepatic hydroxyl-

proline and DNA content) and histopathologically by image analysis and Ishak score. The activity of ALT and AST were significantly elevated while antioxidant enzymes, platelets count, DNA content were significantly decreased from the 8th to the 12th weeks compared to the control. Absence of ANA, ASMA and AMA or LKM were found at different ages. At the 12th week, the Ishak score was score 1 and by image analysis, the area occupied by collagen fibers was 6.24 ± 0.987. A positive correlation was found between biochemical liver fibrosis markers (serum collagenase and hepatic hydroxylproline); DNA content and area occupied by collagen fibers as detected by image analysis. These data, ultimately lead to a new fibrosis index equation.

INTRODUCTION

Iron is a necessary element for all living cells. Iron deficiency with or without anemia is the main cause of nutritional deficiency for human beings. With all the justified awareness of iron deficiency, attention should also be given to the potentially damaging effects of prolonged and indiscriminate iron administration (Lancu et al., 1987). Excess iron deposited chronically in hepatic parenchymal cells is associated with hepatic injury, fibrosis, and ultimately cirrhosis (Powell et al., 1980). These pathological changes occur in both hereditary hemochromatosis (HHC) and in the various forms of secondary hemochromatosis (Bacon et al., 1983).

The role of iron in the pathogenesis of liver injury was demonstrated by Mackinnon et al. (1995) and Siah et al. (2006). They suggested that iron-induced membrane lipid peroxidation occurs in vivo in chronic hepatic iron overload. Iron-induced peroxidative damage may occur via a number of possible mechanisms. In conditions of iron overload, the ability of the hepatocyte to maintain iron in the nontoxic protein-bound ferric state may be exceeded, resulting either in small amounts of ferrous iron or in excessive amounts of low molecular weight chelate iron in the cytosol (Jacobs, 1977). Iron in these forms may play a role in the generation of free hydroxyl radicals by catalyzing the reaction of superoxide radical with hydrogen peroxide. This iron-dependent reaction has been postulated as a

feasible mechanism of hydroxyl radical-induced lipid peroxidation in vivo (Fong et al., 1973 and Halliwell, 1982). Alternatively, it has been suggested that free ferrous iron can serves as a direct initiator of membrane peroxidation. Two proposed mechanisms whereby free ferrous iron could initiate lipid peroxidation are the formation of perferryl ion, FeO_2^{2+}, or the formation of a ternary free radical complex between arachidonic acid, ferrous iron, and oxygen, resulting in peroxidation of the hydrocarbon chain (Kornbrust and Mavis, 1980).

The present study was undertaken to investigate the parallism between biochemical, histochemical and immunological parameters in liver injury model induced by carbonyl iron in an attempt to reach to a new fibrotic index equation.

MATERIALS AND METHODS

Experimental Study

*** Experimental Animals :**

All experiments were performed with adult male Sprague- Dawley rats purchased from Urology and Nephrology center Mansoura University Mansoura Egypt. Forty-two rats were housed in polyethylene cages (5 rats/ cage) with stainless steel wire tops and were allowed commercial standard diet and water ad-libitum. Rats were housed under standard laboratory conditions (room temperature 22 ± 2 ^{o}C, humidity 55± 5L, 12 hours light/dark cycle).

*** Experimental design :**

The healthy rats with an average body weight 240-300 gm were divided into two groups; control (n= 12) and experimental (iron treated rats) (n=30) groups. Iron induced hepatic fibrosis is performed by feeding of the rats with carbonyl iron (3% wt/ wt) for 24 hrs, 72 hrs, one week, 4 weeks, 8 weeks and 12 weeks (5 rats/ time).

Carbonyl iron is an extremely pure form of elemental iron, (>98% iron with <0.8% carbon, <0.3% oxygen, and <0.9% nitrogen). Carbonyl iron (SF- special grade) was purchased from the GAF Corporation (New York). The chow dietary carbonyl iron was prepared by adding 3 gm from carbonyl iron to 97 gm of normal chow diet (Stal et al., 1995).

Biochemical assays :

At the assigned time blood were taken from the tail and liver and spleen samples (5 treated and 2 control rats at each time) and were subjected to the following biochemical investigations:

i- Determination of serum transaminase activities : Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed using a commercially available assay kit (Egyptian American Company for Laboratory Services, Egypt).

ii- Antioxidant enzymes : Superoxide dismutase activity (SOD) in liver homogenates was determined by the procedure of Nishikimi et al. (1972). Reduced glutathione (GSH) was determined by the method of Beutler et al. (1963). This method utilized metaphosphoric acid for protein precipitation and the water soluble 5, 5`-dithiobis (2-nitrobenzoic acid), DTBB, for colour development. Catalase activity (CAT) in homogenate was determined according to the method of Bergmeyer, (1974).

iii- Determination of serum collagenase activity : The method used is a modified form of that reported by Mandl et al. (1953). The extent of collagen breakdown was determined by using Moore and Stein (1948) colorimetric ninhydrin method. The liberated amino acids were expressed as micromoles of leucine per 100 ml serum.

iv -Determination of hepatic hydroxyproline content in liver: The content of hepatic hydroxyproline (as a marker for liver fibrosis) was determined by using the modified method of Laitinen et al., (1974) and Woessner (1961).

v- Determination of DNA content in liver: The determination of DNA in the liver tissue was determined in the nucleic acid extract using the diphenylamine procedure (Disch and Schwartez, 1937).

vi- Manual platelet count of blood (Lewts et al., 1979).

Immunological stud y:

i- Indirect immunofluorescence study: the Kallested HEp-2 kit (BIO-RAD) was used to detect autoanti-

bodies (Anti-nuclear antibodies (ANA), anti-mitochondrial antibodies (AMA) and anti-smooth muscle antibodies (ASMA) were investigated by Indirect Immunofluorescence (IFL) on HEp-2 cells by Evans blue stain (Wasmuth et al., 2004). Autoantibodies in a test sample bind to antigens in the substrate. Washing removes excess serum from the substrat. Fluorescein conjugated (FITC) antiserum added to the substrate attaches to the bound autoantibody. After a second washing step to remove excess conjugate, the substrate is coverslipped and viewed for fluorescent patterns with a fluorescent microscope. Observation of a specific fluorescent pattern(s) on the substrate indicates the presence of autoantibodies in the test sample Bottazzo et al. (1976) and Cassani et al. (1985). The provided kit is supplied with positive controls (ANA, AMA and ASMA Positive Controls) and negative control vials. Positive and negative control samples were prepared for comparison.

ii- Isolation, count and viability of lymphocytes from spleen : Lymphocytes were isolated from spleen according to the method of Weaver and Cross (Weaver and Cross, 1981).The viability of lymphocytes was checked by Trypan blue according to the method of MacLimans et al. (1957).

iii- Detection of autoantibodies: To detect the presence of ANA, ASMA and AMA or LKM autoantibodies in the serum of treated rats, lymphocytoxicity assay was carried out according to the method of Colley et al. (1979).

Histopathological studies :

At the assigned time, the rats were sacrifices under ether anesthesia. The livers were rapidly removed. The specimens were fixed in 10% phosphate buffered formalin, processed by routine histological procedures, dehydrated and cleared in xylene, then, embedded in paraffin, cut at 6 om and stained with Haematoxylin & Eosin stain (H & E) for routine morphological changes, Masson's trichrome stain for connective tissue collagen fibers (Bancroft and Gamble, 2002) and Perls' Prussian blue stain (Culling et al., 1966) for haemosiderin granules.

Fibrosis grade

The grade of liver fibrosis in the

liver sections of all animals were estimated according to modification of HAI score of Ishak et al., 1995.

Image analysis of the area occupied by collagen fibers

Quantitative assessment of liver fibrosis was performed with morphometry on sections processed with Masson's trichrome stain, which specifically stains collagen fibers. (James et al. 1986). The data were obtained using Leica Qwin 500 image analyser computer system (England). The image analyzer was first calibrated automatically to convert the measurement units (Pixels) produced by the image analyser program into actual micrometer units. Using the measurement menu (the area, area %) and standard measuring frame of a standard area equal to 763882 ๐m2 were chosen from the parameters. In the chosen field the Masson's trichome stained areas enclosed inside the standard measuring frame were measured. These measurements were done using an objective lens of magnification 4. The % of the fibrosis area over the whole observed field was assessed to represent the degree of hepatic fibrosis. Several readings were obtained in each specimen (6 slides per animal and at least ten random fields was measured in each slide) (Muller et al. 1988).

Statistical analysis :

The data was represented as means ±SD. The data obtained were subjected to statistical analysis using paired-sample T test. The significant level was set at $P > 0.05$.

RESULTS

Biochemical Assays :

i- Serum transaminases activity:

To measure the degree of liver injury in each stage of the study, ALT and AST parameters were studied in the serum of treated rats. The statistical analysis and mean value of serum ALT and AST in the control and iron-induced liver injury of male rats were represented in Table (1). The mean value of serum ALT and AST at 4, 8 and 12 weeks displayed a very highly significant ($p > 0.001$) increase when compared to that of control group.

ii- Antioxidant enzymes :

Table (2), represents the antioxidant enzymes in liver of control and

iron-treated groups. The mean value of hepatic SOD, GSH activity and CAT in the homogenate liver samples obtained from iron-treated group were highly significantly ($p > 0.001$) decreased from 4th week to the 12th week as compared to that of control group.

iii- Liver fibrosis markers (serum collagenase activity and hepatic hydroxyproline) and DNA content:

The resulted data of liver fibrosis markers and DNA content in control and iron-treated groups (Table 3), showed that there were a very high significant ($p > 0.001$) increase in the concentration of hepatic hydroxyproline and collagenase activity While, there was a highly significant ($p > 0.001$) decrease in the content of hepatic DNA at 4th, 8th and 12th weeks.

iv- Platelets count of blood :

Table (4), display the platelets count (1000/μL) in blood of control and iron-treated groups. The mean count of blood platelets samples obtained from iron-treated group showed that, a very highly significant ($p > 0.001$) decrease at times 4, 8 and 12 weeks as compared to that of control group.

Immunological study :

i- Indirect immunofluorescence study :

The result of fluorescence microscopic investigation of substrate slides stained by Evans Blue, displayed that, there was no detection of autoantibodies in the serum of all rats of treated and control rat groups at 24, 72 hours, 1, 4, 8 and 12 weeks, where there was no specific patterns of apple-green fluorescence are observed (Fig. 1A). The positive control slides of autoantibodies relevant to liver autoimmune were showed in Figures (1B, C and D). The serum was considered positive reaction for ANA, AMA, ASMA and LKM autoantibody

ii- Splenic lymphocytes count and viability:

Splenic lymphocytes count and viability test was performed to indicate that there is no cross reactivity occurred between the lymphocytes and any antibody in the serum of the same treated rats. So, the obtained

results of the mean count of splenic lymphocytes showed no significant change between control and treated rats as shown in Table (5). On the other hand, splenic lymphocytes count showed 100% viability (trypan blue free cells) in all samples of both intoxicated and non-toxicated rat groups in all examination times from 24 hours until 12 weeks.

iii- Detection of Anti-Lymphocytic antibodies :

To detect the presence of autoantibodies, lymphocytes were isolated from spleen of the studied rats in RPMI-media according to the method of Weaver and Cross (1981). The data obtained in Figure (2A) showed that there was no reaction occurred between serum autoantibodies and isolated splenic lymphocytes in both treated and control rat groups (from 24 hours until 12 weeks). Where viable lymphocyte cells were not accept the trypan blue stain. On the other hand the positive control of reaction between serum autoantibodies and isolated splenic lymphocytes of another rat was indicated in Figure (2B), where the lymphocyte cells were dead and thus stained with trypan blue.

Histopathological study :

Haematoxylin and eosin stain :

Sections of control liver showed intact liver architecture with the characteristic lobular pattern. The lobules were roughly hexagonal in shape, with portal triads at the vertices and a central vein in the middle (Fig. 3B).

In treated groups from 24hrs to 4 weeks, the liver sections showed almost normal liver architecture (Fig. 3C). From 8-12 weeks the hepatic tissues showed normal liver architecture but few of the hepatocytes showed large and small empty vacuoles within its cytoplasm. Hepatic steatosis was mainly macrovesicular and panacinar with centrally placed nuclei. Some vacuoles coalesce to form large cysts. Their nuclei were pushed by the accumulated lipid droplet to the periphery of the cells. (Fig. 3A).

Masson's trichrome stain :

Sections of the control livers showed minimal fibrous tissue around portal tracts, central veins and in the wall of the sinusoids (Fig. 4B). From 24 hrs to 8 weeks, the sections were almost like the control (Fig. 4C). At

12th week, fibrosis was seen around the central veins. Thin short septa could be seen extending from most of the portal areas and the central veins into the surrounding parynchyma (Fig. 4A).

Perls' Prussian blue :

In sections of the control rat stained with Perls' Prussian blue stain there was no dark blue granular haemosidrin pigment in neither hepatocytes nor kupffer cells (Fig. 5A).

In rats treated for 24 hrs, 72 hrs and 1 week, no haemosidrin pigment could be observed in the hepatocytes nor kupffer cells.

In rats treated with iron for 4 weeks there were some dark blue granular haemosidrin pigment in the kupffer cells (Fig. 5B).

In treated rats at 8 and 12 weeks there was moderate diffuse haemosidrin pigment; dark blue granular pigment in kupffer cells and in the cytoplasm of the hepatocytes (Fig. 5C and 5 D).

Image analysis of the area occupied by collagen fibers :

Image analysis of Masson's trichrome stained sections of the control rats revealed that the area occupied by collagen fibers was 3.128 ± 0.781.

The area occupied by collagen fibers was significantly increased with administration of carbonyl iron after 1, 4 and 8 weeks (3.828 ± 0.844, 40365 ± 1.16 and 4.712 ± 0.797 respectively) as compared with the control.

At the 12th week of iron treatment the area occupied by collagen fibers was very high significant increased (6.24 ± 0.987) in comparison with the control.

The grade of liver fibrosis :

According to Ishak score, the grade of liver fibrosis increased (grade 1) in animals treated with carbonyl iron for 8 and 12 weeks as compared with controls (Figs. 3 and 4).

Correlation between biochemical and histopathological studies:

Data obtained from parameters of biochemical and histopathological studies indicated that there was a correlation or parallism between the two

studies in iron-treated groups. A correlation between liver fibrosis markers (serum collagease activity and hepatic hydroxyproline) and DNA content and the area occupied by collagen fibers as detected by image analysis had also been found.

From data obtained from fibrosis markers (hydroxyproline concentration and collagenase activity) and data obtained from DNA content we distinguished a new fibrosis index biomarker equation:

Fibrosis index = (hydroxyproline content / collagenase activity) / DNA content = (μg/ml/ Units/ μg/106 cells)

After comparing the data obtained from this equation (Table 6), there was a positive correlation between them, so from this new equation we can determine the fibrosis stage in the liver without the need for liver histopathological investigation.

The Ishak Modified HAI Score (Ishak et al., 1995)

Change	Score
No fibrosis	0
Fibrous expansion of some portal areas, with or without short fibrous septa.	1
Fibrous expansion of most portal areas, with or without short fibrous septa.	2
Fibrous expansion of most portal areas, with occasional portal to portal (P-P) bridging.	3
Fibrous expansion of portal areas, with marked bridging (p-p) as well as portal-central (P-C) bridging.	4
Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis).	5
Cirrhosis probable or definite.	6
Maximum possible score.	6

Image analysis of the area occupied by collagen fibers

Table (1): ALT&AST activities in control and iron-induced liver injury of male Sprague-Dawley rats.

Duration of treatment	ALT IU/L	AST IU/L
Control	37.2±8.3	73.1±6.2
24 hours	37.2±10.8	72..20±3.7
72 hours	31.8±8.37	69.2±7.1
one week	40.6±5.31	78.8±11.5
4 weeks	103.6±5***	144±12***
8 weeks	164.4±6***	225±16***
12 weeks	177.6±8***	268±23***

Results are represented as mean value of five treated rats and two controls in each time ± SD

(*) Significant (P < 0.05), (**) highly significant (P < 0.01) and (***) very highly significant (P < 0.001) when compared to control rats.

Table (2): Antioxidant enzymes in control and iron-induced liver injury of male Sprague-Dawley rats.

Duration of treatment	SOD (U/mg protein)	GSH (nmol/mg protein)	CAT (kU/mg protein)
Control	2876.3±84.3	19.6±0.7	2.16±0.06
24 hours	2841.3±57.43	19.4±0.26	2.23±0.103
72 hours	2783.1±41.88	19.32±0.15	2.21±0.008
one week	2662.3±30.4	19.21±0.28	2.15±0.007
4 weeks	2239.9±60.7***	15.82±0.27***	1.83±0.010***
8 weeks	1992.2±55.9***	15.42±0.414***	1.527±0.012***
12 weeks	1481.7±22.4***	14.32±0.46***	1.03±0.008***

Results are represented as mean value of of five treated rats and two controls in each time ± SD

(*) Significant (P < 0.05), (**) highly significant (P < 0.01) and

(***) very highly significant (P < 0.001) when compared to control rats.

Table (3): Liver fibrosis markers and DNA content in control and iron-induced liver injury of male Sprague-Dawley rats.

Duration of treatment	Serum Collagenase (Units)	Hepatic Hydroxyproline (μg/ml)	DNA content in the liver (μg/10^6 cells)
Control	0.04382±0.008	1.12±0.14	0.044±0.009
24 hours	0.04480±0.001	1.12±0.06	0.044±0.002
72 hours	0.04583±0.001	1.14±0.07	0.043±0.003
one week	0.04752±0.003	1.16±0.03	0.042±0.003
4 weeks	0.05659±0.002***	1.41±0.03***	0.020±0.001***
8 weeks	0.06317±0.003***	1.64±0.02***	0.019±0.002***
12 weeks	0.07563±0.003***	1.80±0.04***	0.008±0.001***

Results are represented as mean value of five treated rats and two controls in each time ± SD

(*) Significant ($P < 0.05$), (**) highly significant ($P < 0.01$) and (***) very highly significant ($P < 0.001$) when compared to control rats

Table (4): The platelet count in control and iron-induced liver injury of male Sprague-Dawley rats.

Duration of treatment	Platelets (1000/μL)
Control	1062.2±103
24 hours	1021±72
72 hours	1023±96
one week	1002.2±59
4 weeks	819.2±12***
8 weeks	712.8±60***
12 weeks	713.8±68***

Results are represented as mean value of five treated rats and two controls in each time ± SD

(*) Significant ($P < 0.05$), (**) highly significant ($P < 0.01$) and (***) very highly significant ($P < 0.001$) when compared to control rats.

Table (5): Splenic lymphocytes (10^6 /ml) count and percentage of viability of non-toxicated and different toxicated male Sprague-Dawley rat groups.

Time of treatment	Count of lymphocytes (10^6 /ml)
Control	13.45±0.5
24 hours	13.69±0.5
72 hours	13.69±0.5
one week	13.69±0.5
4 weeks	13.69±0.5
8 weeks	13.69±0.5
12 weeks	13.69±0.5

The results are expressed as mean of 5 rats / time pointes ± SD.

Table (6):- Morphometric quantitative measurements of liver fibrosis in control and iron treated liver tissues of rats exposed at different intervals and stained with mason's trichrome stain.

Time of Treatment	Area %		
	Min.	Max.	M±SD
Control	2.296	4.912	3.128 ± 0.781
72 hour	0.236	6.176	2.553 ± 2.024
One week	0.081	3.054	3.828 ± 0.844 *
4 weeks	1.141	5.614	4.365 ± 1.16**
8 weeks	0.398	2.645	4.712 ± 0.797 **
12 weeks	4.134	0.987	6.24 ± 0.987***

Area %: represent the degree of hepatic fibrosis, the results are represented as mean value± SD:-

(*) significant at ($p< 0.05$), (**) highly significant at ($p< 0.01$))***) very highly significant at ($p< 0.001$).

Table (7): The relationship between the area occupied by collagen fibers (as detected by image analysis) and the fibrosis index at different durations of treatment with carbonyl iron.

Duration of treatment	Fibrosis Index (μg/ml/ Units/ μg/10^6 cells)	Area occupied by collagen fibers
Control	580.88	3.128 ± 0.781
72 hour	578.47	2.553 ± 2.024
One week	581.2	3.828 ± 0.844 *
4 weeks	1245.8***	4.365 ± 1.16**
8 weeks	1366.4***	4.712 ± 0.797 **
12 weeks	2975***	6.24 ± 0.987***

Hydroxyproline/Collagenase/DNA is the fibrosis index of ratio hepatic hydroxyproline concentration: serum collagenase activity: hepatic DNA content. (*) significant at ($p< 0.05$), (**) highly significant at ($p< 0.01$))***) very highly significant at ($p< 0.001$).

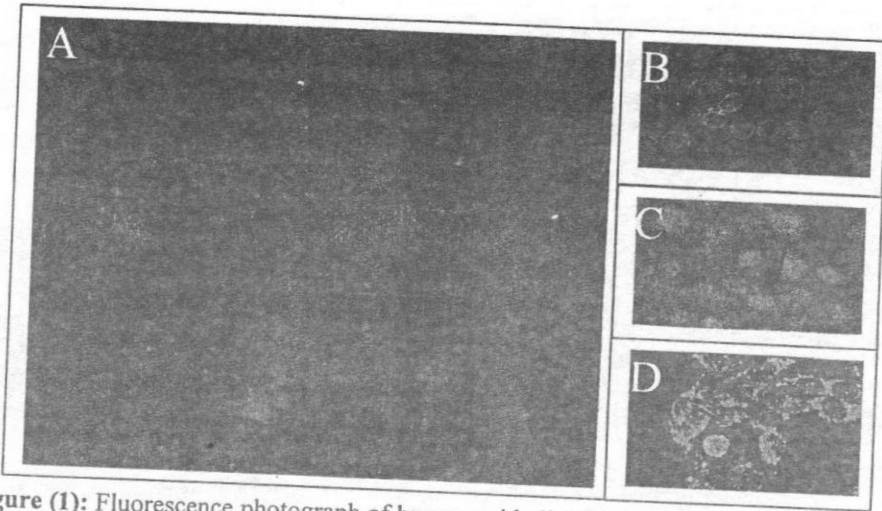

Figure (1): Fluorescence photograph of **human** epithelial (HEp-2) cell lines. 1**A.:** There is no specific patterns of apple-green **fluorescence** is observed on any part of the substrate. The serum is considered **negative** reaction for ANA, AMA, ASMA and LKM autoantibody. **1B:** Control positive reaction for ANA autoantibody. **1C:** Control positive reaction for ASMA autoantibody. **1D:** Control positive reaction for AMA and LKM **autoantibody**. Figures 1B, 1C and 1D are used for comparison **(Evans Blue stain, X40).**

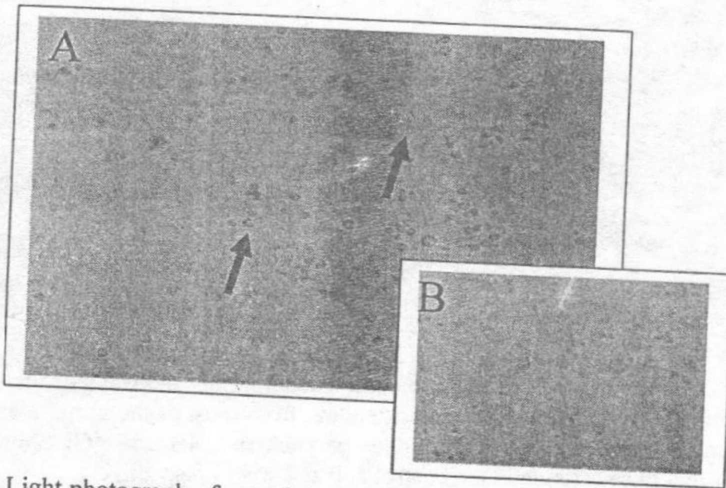

Figure (2): Light photograph of rat spleen **lymphocytes** (↑). **2A:** The lymphocytes were viable and did not accept trypan **blue stain**. The serum is considered negative reaction for autoantibody. **2B:** Control positive reaction for autoantibody, the lymphocyte cells were died and **accept** the trypan blue stain **(Trypan Blue stain, X40)**

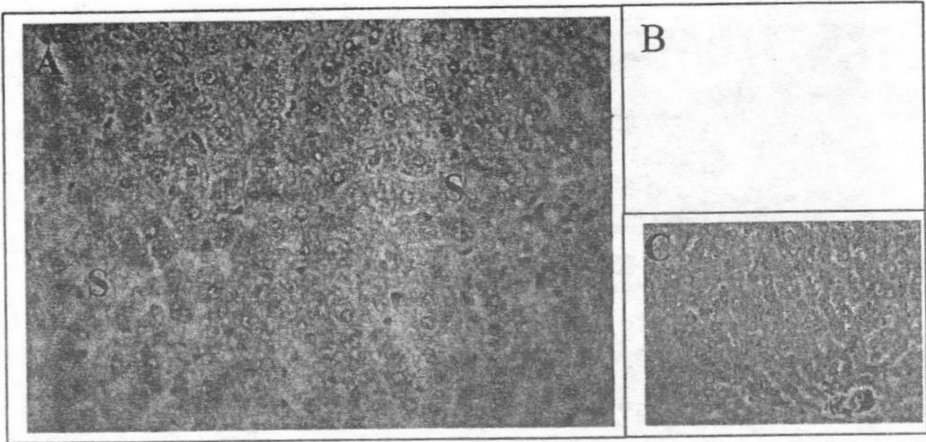

Figure (3): 3A: Light photograph of liver section of iron-treated rats (12 weeks) showing normal liver architecture, few hepatocytes showed large and small empty vacuoles with mild steatosis(S). **3B** and **3C:** Shows almost normal liver architecture in the control and at 4 weeks respectively.

Hematoxylene and Eosin stain X 100

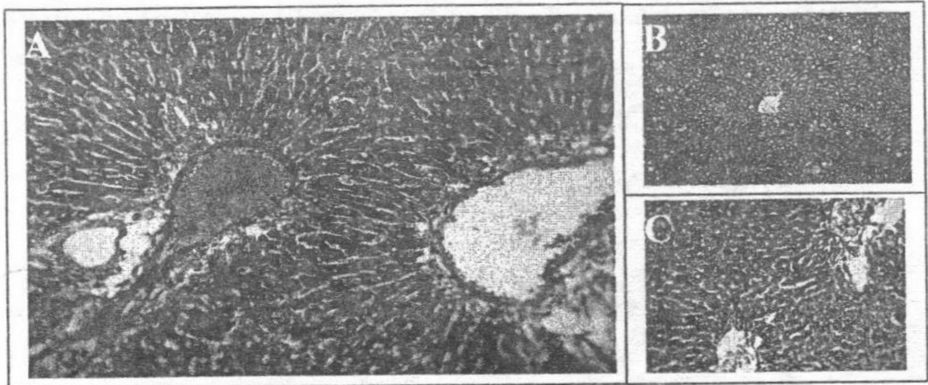

Figure (4): 4A: Light photograph of a liver section of iron treated rat (12w) showing fibrosis around the central veins and extending into the walls of the sinusoids. Thin short septa could be seen extending from most of the portal areas and the central veins into the surrounding parynchyma. **4B** and **4C:** Shows almost normal fibrous tissue in the control and at 4 weeks respectively.

Masson's trichrome X 100

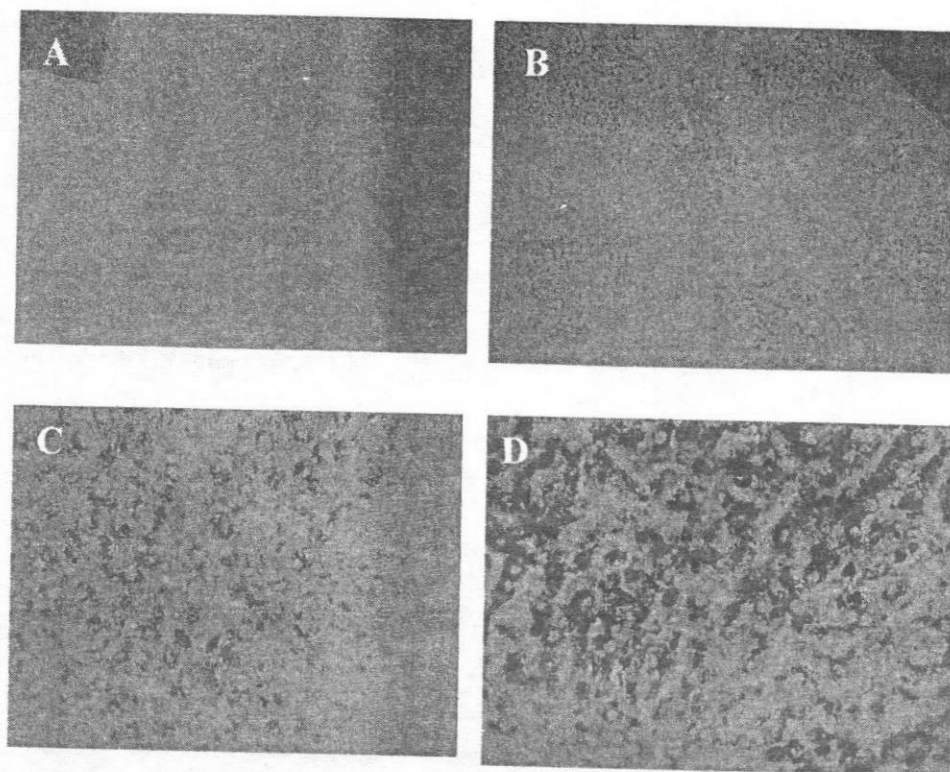

Figure 5: A: Light photograph of a liver section of control rat shows no dark blue granular haemosidrin pigment in neither hepatocytes nor kupffer cells. **B:** Shows some dark blue granular haemosidrin pigment in the kupffer cells. **C** and **D**: At 8 and 12 weeks respectively, showing moderate diffuse haemosidrin pigment in kupffer cells and in the cytoplasm of the hepatocytes.

Perl's Prussian blue stain X 100

DISCUSSION

Carbonyl iron liver injury model :

Loading with high doses of carbonyl iron, a form of iron with higher bioavailability than other forms, has been used in this study most to assess the effects of dietary iron overload in experimental animals. Chronic, high-dose supplementation in rats and mice results in rapid hepatic iron deposition, with a pattern similar to that seen in human HHC. In the presence of available cellular reductants, iron may act as a catalyst in the initiation of free radical-mediated reactions. The resultant oxyradicals or lipid hydroperoxides have the potential to damage a variety of cellular structures, including lipid in organelle membranes, nucleic acid, proteins, and carbohydrates, which could result in the disruption of numerous cellular functions (Ozguner and Sayin, 2002).

The oral carbonyl iron (3% wt/ wt.) used in the current study exhibited biochemical and pathological liver injury criteria at 4 weeks. This result may be comparable with, or even better than, those in previous reports by others (Lancu et al., 1987; Bacon et al., 1983 and Stal et al 1995).

Hepatotoxic agents such as iron, causes hepatic damage with a marked elevation in serum levels of aminotransferases enzymes (AST and ALT) at 4 weeks because these enzymes are cytoplasmic in location and are released into the blood after cellular damage(Mackinnon et al., 1995). In agreement with this investigation, our results showed that a significant increase in the activities of ALT, AST.

William et al. (1997) described the cause of increase in activities of these enzymes in plasma to be due to hepatocellular damage by hepatotoxic agents used in this study. A concentration gradient exists for enzymes between the hepatocyte and the sinusoidal space. On damage to the process of cellular energy production, permeability of the hepatocyte membrane increases and cytosolic isoenzymes of the aminotransferases (ALT and AST) spill into the sinusoids and then the peripheral blood. Permeability of mitochondrial membranes may also increase and mitochondrial isoenzymes are then released as well.

The present study showed that iron produced marked oxidative impact as evidenced by the significant decreases in hepatic Superoxide dismutase, catalase activity, and glutathione content at 4 weeks .Similar results were reported with iron liver toxicated models. Gebhardt, (2002); Pietrangelo, (2003); showed that significant decrease in antioxidant enzymes were demonstrated at 4 weeks in toxicated rats as compared to control rats. From these results it can be concluded that, antioxidant enzymes was able to defend against oxidative stress and protect the cells from potentially harmful attack until 4 weeks but after that the defense decreased.

From the present study (Table 3); it has been found that hepatic hydroxyproline concentration was very significantly increased in the hepatic of toxicated rats with fibrosis than that of untoxicated group. Tanabe et al. (1991) reported that the concentration of L-hydroxyproline in mice female liver increased rapidly during the weeks from (8-11) of schistosoma mansonai infestation. Similarly, increased concentration of hydroxyproline in liver was also reported by others (Weng et al., 2002 and Senthilkumar and Nalini 2004).

The present study showed that, the levels of collagenase in sera of toxicated rats was significantly increased compared to control group (Table 3). These results agree with the results of Maruysma et al (1982) who reported that, the activities of the enzymes that can degrade the extracellular (ECM) matrix were increased with fibrosis. However, hepatic collagenase activities against collagens type I and IV were diminished once liver cirrhosis was established (Maruysma et al (1985 and Miura1985).

Apoptotic cell death is a process frequently occurring in toxin-induced liver injury, depending on the dose and rout of toxin administration, on the time of experimentation and on the assay used for apoptosis detection (Theocharis et al., 2001).

In this study, we detected apoptotic process, using DNA content assay where DNA content increase with increasing cell division and decreased with increasing cell death.

The DNA content of homogenate liver samples obtained from iron-treated rats was very highly significantly decreased at 4 weeks compared to control group. Our results agree with Shi et al., (1998), how demonstrated that apoptosis in hepatocytes was found as early feature of toxicated liver injury.

The present investigation demonstrated that platelets counts were significantly decreased in blood circulation of iron group at 4 weeks compared to control rats (Table 4). This data agrees with an earlier work by Eipel et al. (2004) who reported that platelets were decreased in rat model of systemic endotoximia.

Platelets, arise from giant precursor cells (megakaryocytes) that reside and mature within the bone marrow, undergoing eventual cellular dissolution as they give rise to hundreds of individual platelets. Brass, (2005) proposed that total protein content in toxicated liver decreased so megakaryocyte proteins decreased also, as well as the formation of megakaryocytes cells in bone marrow and differentiated into mature platelets also decreased.

This work was designed to assess the parallism studies between immunological, biochemical and histochemical. There was no prevalence of autoantibobies in the serum of treated rats and there is no significant change between treated and control rats in the titre of autoantibodies. This observation indicates that iron has no immunogenic effect on treated rats over 12 weeks. Where the basis of the immunogenicity may lies in the form of antigenic recognitions. These results are comparable with Lohse et al. (1990 and 1992) who reported that experimental autoimmune hepatitis could not be induced in Lewis rats. In addition to the previous work of Smialowicz et al. (1991) who demonstrated that hepatotoxic agents is not immunotoxic in the rats at dosages that produce overt hepatotoxicity.

The obtained results described immunoreactivity of expected induced autoantibodies with auto-antigen (splenic lymphocytes) of the same rat at different time's intervals, using in vitro lymphocytotoxicity assay. These results indicate that there is no in vitro reactivity between serum autoantibodies and isolated splenic lympho-

cytes in both Iron-treated and control group over 12 weeks. These observations indicate the absence of hepatitis autoantibodies from the serum of toxicated rat groups.

Interestingly, the current results showed that iron molecule failed to stimulate immune system of rats to secreate autoantibodies. This result is in agreement with the results of Bahia-Oliveira et al. (1992) and Hirsch et al. (1997) who reported that nonimmunized rats did not develop significant levels of hepatitis autoantibodies.

Histpathological evaluation revealed mild steatosis, Hepatic steatosis was mainly macrovesicular and panacinar with centrally placed nuclei. Some vacuoles coalesce to form large cysts. Their nuclei were pushed by the accumulated lipid droplet to the periphery of the cells.

The macrovesicular steatosis, probably results from increased uptake and synthesis of fatty acids in the liver (Burt et al., 1998). Adipose tissue becomes resistant to insulin, serum lipoprotein levels shift, and flux of free fatty acids to the liver increase (Campbell et al., 1994 and Gibbons et al., 2000). The cumulative effects of insulin resistance and increased circulating free fatty acides act in concert to channel fatty acides into storage rather than secretory and degradative pathways (Gibbons and Wiggins, 1995).

Analysis of the liver sections stained with Masson's trichrome stain revealed a progressive increase in the amount of fibrous tissue with increasing the duration of carbonyl iron treatment. At 12th week, fibrosis was seen around the central veins. Thin short septa could be seen extending from most of the portal areas and the central veins into the surrounding parenchyma.

Liver fibrosis is created not only as a consequence of the changes in the secretion of matrix, but also from changes in its degradation, which means a loss of the dynamic functional balance between fibrogensis and fibrolysis (Arthur, 2002). During the development of fibrosis, the capacity of the degradation is not eliminated, but is reduced (Batallar and Brenner,

2001). On this basis, the increase in the amount of the collagen fibers could be the net result of two different processes formation of new fibers and degeneration of the already formed ones. With continous and repeated injury, the formation of new fibers predominated and the degradation of the already formed fibers decreased.

In the present study, moderate diffuse haemosidrin pigment were seen in the kupffer cells and in the cutoplasm of the hepatocytes. Morphometric measurement of the area and area % of the amount of fibrosis was increased compared to control group. These findings are in agreement with Pietrangelo, 2003; Petersen, 2005 and Novo et al., 2006.

Gualdi et al. (1994) suggested that either iron loaded hepatocytes directly release profibrogenic substances, which activate hepatic stellate cells or release substances which stimulate Kupffer cells to produce profibrogenic substances which activate hepatic stellate cells. Iron overload can induce lipid peroxidation of organic membranes leading to cell injury and cell death (Bacon et al., 1983; Britton et al., 1990; Younes and Wess, 1990 and Dabbagh et al, 1994). Lipid perioxidation products have been shown to stimulate collagen production in activated hepatic stellate cells and cultured human fibroblasts (Leonarduzzi et al., 1997).

Although the grade of fibrosis at the 12th week did not exceed score 1 according to Ishak score (Ishak et al., 1995), a significant correlation was found between fibrosis index and the area occupied by collagen fibers. (Tab.6). This correlation demonstrates that, we can determine the fibrosis stage in the liver by using the new fibrosis index biomarker without making liver histochemical investigation. Therefore, this fibrosis index biomarker may be used as a useful biomarker in the diagnosis and follow up of liver diseases patients.

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الملخص العربى

التغيرات البيوكيميائية و المناعية و الهستوباثولوجية لكبد الفأر المستحث بمركب الحديد الكربونى

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يعتبر الحديد العنصر الأساسى لجميع الخلايا الحية وان نقصه المنفرد أو المصحوب بأنيميا من العوامل الأساسية للنقص الغذائى للجسم البشرى على النقيض من ذلك فأن تراكم الحديد فى الخلايا يسبب صور عديدة من الأمراض من أهمها داء ترسب الأصبغة الدموية الوراثى he-reditary hemochromatosis (HH)) . لذلك فقد أهتمت الدراسة الحالية بقياس وفحص التغيرات بين المقايس البيوكيمائية و المناعية و الهستوباثولوجية فى كبدالفئران المستحثة بمركب الحديد الكربونى و مدى امكانية استخدمها كمؤشر لقياس درجة التليف الناشئى فى خلايا الكبد المستحثة بمركب الحديد الكربونى و لقد اجريت تلك الدرا سة على عدد ٤٢ فار مقسمة الى مجموعتين : مجموعة كنترول مكونة من ١٢ فار و مجموعة مكونة من ٣٠ فار قد تمت معالجتها بمركب الحديد الكربونى فى الغذاء بجرعة مقدرها ٣% على فترات زمنية مختلفة (٢٤ ساعة , ٧٢ ساعة , اسبوع ٤. أ سابيع , ٨ أسابيع , ١٢ أسبوع) ولقد أخذت عينات الدم و عينات أنسجة الكبد لمجموعتى الفئران الكنترول و المعالجة بالحديد الكربونى عند الفترات الذمنية قيد الدراسة. و لقد تم قياس كفائة انزيمات الكبد (ALT, AST) و انزيمات المضادة للأكسدة (SOD,CAT,GSH) و عدد الصفائح الدموية و الأجسام المضادة التلقائية (ANA,ASMA,AMA or LKM) فى عينات السيرم . اما عينات انسجة الكبد فقد تم

صباغتها بالصباغات الهستوباثولوجية القياسية المختلفة (الهيماتوكسالين و الأيوسين و الماسون ترايكروم و البيربروسين الأزرق) و ذلك لقياس الحالة المرضية الناشئة من مركب الحديد الكربونى و لقد تم ترتيب درجة تليف خلايا الكبد بأستخدام معاملات التليف البوكيمائية مثل (البروكولاجين فى السيرم , و الهيدروكسيبرولين و محتوى الحامض النووى) و الهستوباثولوجية بأستخدام نظام تحليل الصور و معامل ترتيب التليف للعالم ايزاك (Image analysis and Ishak Score). و لقد أظهرت الدراسة ارتفاع ملحوظ فى انزيمات الكبد وانخفاض فى منسوب كل من الأنزيمات المضادة للأكسدة و عدد الصفائح الدموية وكذلك منسوب الحامض النووى وغياب الأجسام المضادة التلقائية فى الفترة الزمنية من الأسبوع الثامن حتى الأسبوع الثانى عشر فى المجموعة المعالجة بالحديد الكربونى مقارنة بالكنترول. أما عند الأسبوع الثانى عشر فقد أظهرت تحاليل الصور و المعاملات البيوكيمائية لدرجة تليف خلايا الكبد المقاسة فى الدم (pro-collagen, hydroxyproline, DNA) و مقارنتها بمعامل ايزاك الذى يساوى (١) أن درجة تليف خلايا الكبد بمادة الكولاجين تشغل مساحة مقدرها (6.24 ±0.987) ميما يشير الى وجود علاقة وثيقة بين تلك المعاملات و مدى أهميتها التى يمكن استخدمها فى تحديدو قياس درجة تليف خلايا الكبد .