Parallelism Study between Biochemical, Immunological and Histochemical Parameters of Liver Injury Induced by Carbon Tetrachloride on Rats

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Abstract: This study was carried out to investigate, the parallelism between biochemical, histochemical and immunological parameters in liver injury model induced by CCl4. We evaluated, (a) The activities of serum aminotransferases (ALT, AST), blood platelets count, hepatic antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), hepatic hydroxyproline concentration, serum collagenase activity and hepatic DNA content. (b) Serum autoantibodies (ANA, ASMA and AMA or LKM). (c)Liver histology in control and CCl4-treated rats at different interval times 24, 72 hrs and 1, 4, 8 and 12 weeks. The activities of ALT, AST, collagenase and hydroxyproline concentration, were significantly elevated, while hepatic antioxidant enzymes, blood platelets count and hepatic DNA content were decreased in intoxicated rats as compared to non-toxicated rats at 24-72 hrs. On histological examination, steatosis, lymphocyte inflammation and fibrosis were illustrated at 24-72 hrs in CCl4-treated rats compared to control rats. Absent of autoantibodies from the serum but lymphocytes in tissues were observed began from sample of 72 hrs. Thus we concluded that a parallelism study was demonstrated between biochemical, immunological and histochemical parameters in CCl4-induced liver injury rats.


Key words: Liver Fibrosis, Autoantibodies, Hydroxyproline, CCl4

1. Introduction:
Carbon tetrachloride (CCl4) is one of the most used hepatic toxins for experimental induction of liver fibrosis on rats[1,2]. The mechanism of CCl4-induced hepatotoxicity, especially necrosis and fatty liver, has long been a challenging subject of many researchers from various fields over the past 50 years. Even though the mechanisms of tissue damages are different among chemicals and affected tissues, CCl4 has played a role as a key substance of tissue injury. A number of studies have been conducted and various hypotheses have been raised. As a result, several important basic mechanisms of tissue damages have emerged: involving metabolic activation, reactive free radical metabolites, lipid peroxidation, covalent binding and disturbance of calcium homeostasis[1].

Carbon tetrachloride has been used extensively to study liver injury induced by free radicals in an animal model system. Liver damage (inflammation) caused by CCl4 in mouse model system is closely to analogue of hepatotoxicity in human[3]. The compound is bioactivated by cytochrome P-450-mediated reactions to CCl3, free radicals[4-8], which is further converted to a peroxy radical, CCl3O2[7,8]. The free radicals CCl3 and CCl3O2 readily react with polyunsaturated fatty acids of the endoplasmic reticulum and other hepatocellular membranes to initiate the formation of organic lipid peroxides. In the presence of cellular O2, these organic peroxy radicals in turn can react with other polyunsaturated fatty acids to perpetuate a series of self-propagating chain reactions, known as "propagation of lipid peroxidation"[10].

In addition, it has been shown that CCl4-induced toxicity may stimulate endogenous reactive oxygen and nitrogen species that have also been suggested to play an important role in the pathogenesis of hepatotoxicity[13]. Hierholzer et al.[12] suggested that inducible nitric oxide synthase (iNOS)-generated nitric oxide not only directly contributes to tissue damage but that it also up regulates the inflammatory response through specific signaling mechanisms. Some reports have demonstrated that induced nitric oxide overproduction occurs in the liver of rats with CCl4-induced acute liver injury[13,14] and suggested that iNOS may act as a mediator in the pathogenesis of hepatotoxicity in rats[13,15].

On the other hand, trichloromethyl free radicals can react with sulphydryl groups such as glutathione and protein thiols. The covalent binding...
of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events that eventually leads to membrane lipid peroxidation and finally cell necrosis\(^{(16)}\). Moreover, hepatic glutathione level was decreased after CCl\(_4\) administration due to the reaction of glutathione with CCl\(_4\)-derived free radicals in the hepatocytes\(^{(17,18)}\). Accordingly, CCl\(_4\)-induced hepatotoxicity in rodents was reported to be partially protected by the pretreatment of antioxidants\(^{(19)}\).

2. Material And Methods

Experimental animals:

All experiments were performed with adult male Sprague-Dawley rats purchased from Urology & Nephrology Center Mansoura University, Egypt. 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°C, humidity 55 ± 5L, 12 hours light/dark cycle). Then, these rats were divided into 2 groups.

Group (I): Healthy normal rats (n = 10) fed on a standard chow diet with an average body weight of 240 - 306 g.

Group (II): Carbon tetrachloride (CCl\(_4\)) treated rats (n = 30) weighing (290 - 305 g) were fed on a standard chow diet. The animals were injected intraperitoneal by 50% CCL\(_4\) in corn oil (1.2 ml/kg b.w.) 3 times a week for 12 weeks.

Samples collection:

Blood, liver and spleen tissues samples were tacked for analysis during rats sacrificed at 24 hr., 72 hr., one, 4, 8 and 12 weeks (5 rats/time points). The rats were fasted over night and anesthetized by diethyl ether inhalation (Codex, Carlo Erba, Milan, Italy)\(^{(20)}\).

Biochemical Study:

(i) Determination of Serum Transaminase Activities: Glutamic pyruvate transaminase (GPT); alanine aminotransferase (ALT); and Glutamic oxaloacetate transaminase (GOT); aspartate aminotransferase (AST). Activities were assayed in serum samples by the method of Reitman and Frankel\(^{(21)}\), using a commercially available assay kit (Egyptian American Company for Laboratory Services, Egypt).

(ii) Manual platelet count of whole blood: The method was recommended in preference to that using diluent of 1% aqueous solution of ammonium oxalate in which the red cells were lysed\(^{(22)}\).

(iii) Antioxidant enzymes:

- Superoxide dismutase activity;
- Reduced glutathione and Catalase activity was determined in liver homogenates by the methods of Nishikimi et al.\(^{(23)}\); Beutler et al.\(^{(24)}\) and Bergmeyer\(^{(25)}\), respectively.

(iv) Liver fibrosis markers:

(a) Determination of hepatic hydroxyproline content in liver: The content of hepatic hydroxyproline was determined by using the modified method of Laitinen\(^{(26)}\) and Woessner\(^{(27)}\).

(b) Determination of serum collagenase activity: The method is a modified form of that reported by Mandl et al.\(^{(28)}\) where in collagenase is incubated for 5 hrs with collagen. The extent of collagen breakdown was determined by using Moore and Stein\(^{(29)}\), colorimetric ninhydrin method. The liberated amino acids were expressed as micromoles of leucine per 100 ml serum.

(vi) Determination of DNA content in liver: The determination of DNA in the liver tissue residues was based on the reaction between deoxyribose and diphenylamine. DNA content was determined in the nucleic acid extract using the diphenylamine procedure described by Disch and Schwartez\(^{(30)}\).

Immunological Study:

(i) Indirect immunofluorescence study: The detection and semi-quantitation of autoantibodies aid in the diagnosis of autoimmune diseases. The Kallested HEp-2 kit (BIO-RAD) was used to detect autoantibodies to nuclear (ANA), mitochondrial (AMA) = liver/Kidney micosomal (LKM)} and smooth muscle (SMA) antigens. Anti-nuclear antibodies (ANA), anti-mitochondrial antibodies (AMA), anti-smooth muscle antibodies (SMA), and anti-liver/Kidney micosomal antibodies (LKM) were investigated by Indirect Immunofluorescence (IFL) on HEp-2 cells\(^{(31)}\).

(ii) Isolation of lymphocytes from spleen: Lymphocytes were isolated from spleen of Sprague-Dawley rats according to the method of Weaver and Cross\(^{(32)}\).

(iii) Viability test: The viability of lymphocytes was checked by Trypan blue exclusion according to the method of MacLimans et al.\(^{(33)}\).

(iv) Detection of autoantibodies: To detect the presence of autoantibodies in the serum of treated rats, lymphocytotoxicity assay was carried out according to the method of Colley et al.\(^{(34)}\).
(E) Histochemical study: Formalin fixed tissues were processed routinely, embedded in paraffin wax and 4 µm sections were cut. The sections were stained with Haematoxylin & Eosin stain (35) and fibrosis stages were demonstrated by using special collagen stain Masson’s trichrome stain (36).

3. Results

All intoxicated animals developed liver injury within 24 hours following CCl₄ injection to rats of CCl₄ group. The obtained results were after studies of biochemical, immunological and histochemical parameters over 12 weeks.

Biochemical study:

Table (1): ALT & AST activities and platelets count in non-toxicated and CCl₄-induced liver injury of male Sprague-Dawley rats (M ± SD).

<table>
<thead>
<tr>
<th>Time of intoxication</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>Platelets (1000/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37.2±8.3</td>
<td>73.1±6.2</td>
<td>1062.2±103</td>
</tr>
<tr>
<td>Basal</td>
<td>38.4±9.1</td>
<td>71.6±10.9</td>
<td>1058.4±111</td>
</tr>
<tr>
<td>24 hours</td>
<td>379±14***</td>
<td>890±18***</td>
<td>1105.2±70</td>
</tr>
<tr>
<td>72 hours</td>
<td>204±45***</td>
<td>526.6±22***</td>
<td>895.6±14***</td>
</tr>
<tr>
<td>One week</td>
<td>367±17***</td>
<td>1154±61***</td>
<td>823.4±27***</td>
</tr>
<tr>
<td>4 weeks</td>
<td>309±50***</td>
<td>754.2±71***</td>
<td>720.4±55***</td>
</tr>
<tr>
<td>8 weeks</td>
<td>337±30***</td>
<td>768.6±62***</td>
<td>579.6±66***</td>
</tr>
<tr>
<td>12 weeks</td>
<td>386±14***</td>
<td>902.4±25***</td>
<td>495.8±26***</td>
</tr>
</tbody>
</table>

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

(i) Serum transaminase activities:

The relations between ALT and AST mean value and time was shown in Table (1). A very highly significant (p < 0.001) increase in the mean value of ALT and AST were observed at times from 24 hrs until 12 weeks as compared to non-toxicated rat group.

(ii) Manual platelets count of whole blood:

Table (1), display the platelets count (1000/µL) in blood of non-toxicated and different intoxicated male Sprague-Dawley rat groups. The data shows a very highly significant (p < 0.001) decrease in the mean count of platelets began during the 72 hours in CCl₄-induced rats as compared to that of non-toxicated group.

Table (2): Antioxidant enzymes in non-toxicated and CCl₄-induced liver injury of male Sprague-Dawley rats (M ± SD).

<table>
<thead>
<tr>
<th>Time of intoxication</th>
<th>SOD (U/mg protein)</th>
<th>GSH (nmol/mg protein)</th>
<th>CAT (kU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2876.3±84.3</td>
<td>19.6±0.7</td>
<td>2.16±0.06</td>
</tr>
<tr>
<td>Basal</td>
<td>2887.7±84.3</td>
<td>19.5±0.8</td>
<td>2.17±0.05</td>
</tr>
<tr>
<td>24 hours</td>
<td>2700.8±168.3</td>
<td>19.3±0.01</td>
<td>2.22±0.013</td>
</tr>
<tr>
<td>72 hours</td>
<td>2198.1±83.9***</td>
<td>14.8±0.48***</td>
<td>1.42±0.013***</td>
</tr>
<tr>
<td>One week</td>
<td>1005.9±64.1***</td>
<td>11.65±1.2***</td>
<td>1.03±0.011***</td>
</tr>
<tr>
<td>4 weeks</td>
<td>835±185.1***</td>
<td>8.73±0.5***</td>
<td>0.46±0.011***</td>
</tr>
<tr>
<td>8 weeks</td>
<td>814.4±55.8***</td>
<td>7.83±0.3***</td>
<td>0.318±0.013***</td>
</tr>
<tr>
<td>12 weeks</td>
<td>238.4±95.1***</td>
<td>6.53±0.54***</td>
<td>0.091±0.009***</td>
</tr>
</tbody>
</table>

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

(iii) Antioxidant enzymes:

Table (2), represents the antioxidant enzymes in liver of non-toxicated and CCl₄ intoxicated male Sprague-Dawley groups. The mean value of hepatic SOD, CAT activity and GSH content in the homogenate liver samples obtained from CCl₄-intoxicated group were significantly high (p < 0.001), decreased at 72 hrs as compared to that of non-toxicated group.
(iv) Liver fibrosis markers and DNA content:

Table (3) Liver fibrosis markers and DNA content in non-toxicated and CCl4-intoxicated male Sprague-Dawley rat groups. The resulted data showed that there were a very highly significant (p< 0.001) increase in the concentration of hepatic hydroxyproline and collagenase activity at 72 hours. While, there was a very highly significant (p< 0.001) decrease in the content of hepatic DNA in rats of CCl4-intoxicated group as compared to control group at times from 72 hrs until 12 weeks.

Table (3): Liver fibrosis markers and DNA content in non-toxicated and CCl4-induced liver injury of male Sprague-Dawley rats (M ± SD).

<table>
<thead>
<tr>
<th>Time of intoxication</th>
<th>Hydroxyproline (µg/ml)</th>
<th>Collagenase (Units)</th>
<th>DNA content (µg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.12±0.14</td>
<td>0.0438±0.008</td>
<td>0.044±0.009</td>
</tr>
<tr>
<td>Basal</td>
<td>1.15±0.12</td>
<td>0.0448±0.007</td>
<td>0.045±0.008</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.17±0.03</td>
<td>0.0466±0.003</td>
<td>0.040±0.001</td>
</tr>
<tr>
<td>72 hours</td>
<td>1.92±0.31***</td>
<td>0.0571±0.002***</td>
<td>0.020±0.001***</td>
</tr>
<tr>
<td>one week</td>
<td>2.73±0.02***</td>
<td>0.0586±0.002***</td>
<td>0.019±0.001***</td>
</tr>
<tr>
<td>4 weeks</td>
<td>2.82±0.04***</td>
<td>0.0938±0.001***</td>
<td>0.017±0.002***</td>
</tr>
<tr>
<td>8 weeks</td>
<td>3.14±0.08***</td>
<td>0.1441±0.003***</td>
<td>0.004±0.001***</td>
</tr>
<tr>
<td>12 weeks</td>
<td>3.22±0.03***</td>
<td>0.1547±0.003***</td>
<td>0.003±0.001***</td>
</tr>
</tbody>
</table>

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

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[≤ +1 (-ve results)] of all rats of intoxicated and non-toxicated rat groups at 24, 72 hours, 1, 4, 8 and 12 weeks, where there was no specific patterns of apple-green fluorescence are observed on any part of the substrate (Fig. 1A). The positive control slides of autoantibodies relevant to liver autoimmune serology were showed in Figs.(1B-D). The serum was considered positive reaction for ANA, AMA, ASMA and LKM autoantibody, however apple-green fluorescence were noted as in nucleus membrane (ANA +ve) (Fig. 1B), actin in the microfilaments (ASMA +ve) (Fig. 1C) and numerous cytoplasmic speckles in a fibrous network appearing mainly in the cytoplasm of the cells (AMA and LKM +ve) (Fig. 1D).

(ii) Splenic lymphocytes count:

There was no significant change in the lymphocyte count of intoxicated rats and that of the non-toxicated rats. In the other hand, the microscopic investigation of heamocytometer slide displayed that splenic lymphocytes were not stained by trypan blue, so the viability percentage was 100% in all samples of both intoxicated and non-toxicated rat groups in all examination times from 24 hrs until 12 weeks.
Fig.(1C): Fluorescence photograph of human epithelial (HEp-2) cell lines. Anti-smooth muscle antibodies ASMA (↑) with apple-green fluorescence are shown as a cotton-like diaphanous appearance and reacting with actin in the microfilaments. The serum is considered positive reaction for ASMA autoantibody. (Evans Blue stain, X40).

Fig.(1D): Fluorescence photograph of human epithelial (HEp-2) cell lines. The AMA and LKM patterns (↑) with apple-green fluorescence are identified by numerous cytoplasmic speckles in a fibrous network appearing mainly in the cytoplasm of the cells (Evans Blue stain, X40).

(iii) Detection of autoantibodies:
Fig. 2(A) illustrate the microscopic observation showed that there was no reaction occurred between serum autoantibodies and isolated splenic lymphocytes in both intoxicated and non-toxicated rat groups in all examination times from 24 hrs until 12 weeks. Where the lymphocyte cells were live and did not accept the stain of trypan blue. In the other hand the positive control of reaction between serum autoantibodies and isolated splenic lymphocytes of another rat was indicated in Fig.(2B), where the lymphocyte cells were died and accept the stain of trypan blue.

Fig. (2A): Light photograph of rat spleen lymphocytes (↑). The lymphocytes were viable and did not accept trypan blue stain. The serum is considered negative reaction for autoantibody (Trypan Blue stain, X40).

Fig. (2B): Light photograph of rat spleen lymphocytes (↑). The lymphocytes were died and accept Trypan blue stain. The serum is considered positive reaction for autoantibody. (X40).

Histochemical study:
(i) Liver pathology:
Light microscopy investigation showed that, there was no pathological change observed in hepatic tissues slides of control and at basal time rats in CCl₄ group stained by (Haematoxylin and Eosin) (Fig.3).

(ii) Haematoxylin and eosin stain:
The haematoxylin eosin stains the cytoplasm with red colour; nuclei with blue colour and show the steatosis and lymphocyte infiltration in stained tissue.

Fig.(4-A1) illustrates that, the pathology of hepatic tissues stained by haematoxylin and eosin in CCl₄ injected group at 24 hrs. Where, liver biopsy showed that, hepatocytes in the centrilobular areas present degenerative changes; mild macrovesicular fatty change, and scattered leucocytes infiltrations were also evident in some hepatocytes. Weak leucocytes infiltrations in some hepatocytes and mild steatosis were observed at 72 hours (Fig. 4-B1). As clarified in Figs. (4-C1&D1), there is moderate extensive steatosis; moderate lymphocyte infiltration; and foamy degeneration of hepatocytes at one and four weeks. Moreover, a severe inflammation and severe steatosis were noted at 8 and 12 weeks (Fig. 4E1&6F1) as compared to that of non toxicated group.
(iii) Masson’s trichrome stain:

The Masson’s trichrome stain the nuclei with black colour, the cytoplasm, keratin, muscle fibers and intracellular fibers with red colour and collagen (fibrous tissue), basement membrane and mesangium with blue colour. This stain is special for collagen and shows the degree of fibrosis in stained section.

Light microscopy investigation showed that, there was no pathological change observed in hepatic tissues slides of control and at basal time rats in CCl₄ group stained by Masson’s trichrome (Fig.5).

As shown from Fig. (5-A2), there was no fibrosis investigated in the CCl₄-intoxicated models of hepatic tissues stained by masson’s trichrome at 24 hours. Figs. (6B2-7D2) demonstrates that, the degree of fibrosis at 72 hours, one week and 4 weeks are a weak pericellular fibrosis (a few collagen fibrils extended from the central vein and portal tract. While, in Figs(7-E2&F2), the degree of fibrosis at 8 and 12 weeks are incomplete cirrhosis (collagen fibrils extended into and encompassed the whole lobule as compared to non-toxicated groups.
(D) Correlation between biochemical and histopathological studies:
Data obtained from parameters of biochemical and histopathological studies indicated that there was a correlation or parallism between two studies in the CCl4-intoxicated group. Whereas a directly correlation in each of ALT and AST activities; hydroxyproline concentration; collagenase activity; steatosis; inflammation; necrosis and fibrosis were demonstrated. In addition, antioxidant enzymes activities; platelets count and DNA content were correlated inversely with the previous parameters. All these data confirmed the pathological evaluation as hepatic injury in the CCl4 experimental model over 12 weeks.

(E) Fibrosis index biomarker:
From data obtained from fibrosis marker such as hydroxyproline concentration and collagenase activity and data obtained from DNA content we distinguished new fibrosis index biomarker by making new equation like

\[
\text{Fibrosis index} = \frac{\text{hydroxyproline content}}{\text{collagenase activity}} \times \frac{\text{DNA content}}{\text{Units/} \mu\text{g/}10^6 \text{ cells}}
\]

After comparing between data obtained from this equation and fibrosis stages obtained from histochemical study Table (4). We found that, there was a positive correlation between them, so from this new equation we can determine the fibrosis stage in the liver without making liver histochemical investigation.

Table (4): The stages of the liver fibrosis as histological evaluation and the corresponding rang of fibrosis index value as biochemical parameters

<table>
<thead>
<tr>
<th>Stages of fibrosis</th>
<th>Fibrosis Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/ml/ Units/ µg/10^6 cells)</td>
</tr>
<tr>
<td>S0</td>
<td>(500 - 700)</td>
</tr>
<tr>
<td>S1</td>
<td>(1000 - 2500)</td>
</tr>
<tr>
<td>S2</td>
<td>(2500 - 4500)</td>
</tr>
<tr>
<td>S3</td>
<td>(4500 - 7000)</td>
</tr>
<tr>
<td>S4</td>
<td>&gt; 7000</td>
</tr>
</tbody>
</table>

Hydr./Coll./DNA is the fibrosis index of ratio hepatic hydroxyproline concentration: serum collagenase activity: hepatic DNA content

Correlation between fibrosis index biomarker and fibrosis score in CCl4 group:
A correlation was made between fibrosis index biomarker and fibrosis score evaluated by histochemical study in CCl4-induced liver injury group. Using simple linear regression analysis, a positive correlation was observed between fibrosis index and fibrosis score \(r = 0.90, \text{Tab.}5\).
Table (5): Fibrosis index in non-toxicated and CCl\textsubscript{4}-induced liver injury of male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Time</th>
<th>F. scores</th>
<th>Fibrosis Index (µg/ml/ Units/ µg/10\textsuperscript{6} cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>601.8±9.5</td>
</tr>
<tr>
<td>Basal</td>
<td>0</td>
<td>595.8±8</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>627.2±6</td>
</tr>
<tr>
<td>72 hours</td>
<td>1</td>
<td>1679.4±15</td>
</tr>
<tr>
<td>week</td>
<td>1</td>
<td>2449±17</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1</td>
<td>1768.2±12</td>
</tr>
<tr>
<td>8 weeks</td>
<td>3</td>
<td>5447.2±16</td>
</tr>
<tr>
<td>12 weeks</td>
<td>3</td>
<td>6934.5±18</td>
</tr>
</tbody>
</table>

Results are represented as mean value of five rats in each time ± SD

4. Discussion:

The CCl\textsubscript{4} produces an experimental liver damage, which histologically resembles viral hepatitis. The chronic liver damage induced by carbon tetrachloride in rats produces liver fibrosis and biochemical and histological patterns that resemble human liver cirrhosis\textsuperscript{(37)}. Liver injuries induced by CCl\textsubscript{4} are the distinct symptom of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs\textsuperscript{(38)}.

The rats of the present study injected intraperitonealy by 50% CCl\textsubscript{4} in corn oil (1.2 ml/kg of body weight) 3 times a week for 12 weeks. All injected rats exhibited liver injury criteria, at 24 hrs which are in agreement with the findings obtained by Theocharis et al.\textsuperscript{(39)}; Ko and Lim\textsuperscript{(40)}.

Hepatotoxic agents such as CCl\textsubscript{4} causes hepatic damage with a marked elevation in serum levels of aminotransferases enzymes (AST and ALT) because these enzymes are cytoplasmic in location and are released into the blood after cellular damage\textsuperscript{(40)}. In agreement with this investigation, our results showed that a significant increase in the activities of ALT, AST. William et al.\textsuperscript{(57)} 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, which is dependent on normal cellular metabolism for its maintenance, exists for enzymes between the hepatocytes 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 sinususoids and then the peripheral blood. Permeability of mitochondrial membranes may also increase and mitochondrial isoenzymes are then released as well.

This study demonstrated that platelets counts were significantly decreased in blood circulation of CCl\textsubscript{4} group at 72 hrs as compared to non-toxicated rats. This data agrees with an earlier work by Eipel et al.\textsuperscript{(41)} who reported that platelets were decreased in rat model of systemic endotoximia.

Platelets, on the other hand, 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\textsuperscript{(55)} proposed that 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.

The present study showed that CCl\textsubscript{4} produced marked oxidative impact as evidenced by the significant decreased in the hepatic Superoxide dismutase, catalase activity, and glutathione content. Indeed the results presented confirm the previous works. Ko and Lim\textsuperscript{(40)} and Liu et al.\textsuperscript{(42)} showed that inverse correlations between antioxidant enzymes and pathology scores and / or lipid pero-oxidation have been found in rats with CCl\textsubscript{4}-induced cirrhosis. From these results it can be concluded that, cellular antioxidant enzymes failed to resist the excesses of oxidative stress inside hepatocyte cells at 72 hrs in CCl\textsubscript{4} injected rats.

From the present study; 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.\textsuperscript{(43)} reported that the concentration of L-hydroxyproline in the liver of female mice increased rapidly during the weeks (8-11) of Schistosoma mansoni infestation. Similarly, increased concentration of hydroxyproline in liver was also reported by others\textsuperscript{(44-46)}.

The present study showed that, the levels of collagenase in sera of toxicated rats was significantly increased compared with the level in sera of subjects of the untoxicated control group. These results agree with the results of Maruyama et al.\textsuperscript{(47)} 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\textsuperscript{(48, 49)}.

Apoptotic cell death is a process frequently occurring in toxin-induced liver injury, depending on
the dosage and route of toxin administration, on the time of experimentation and on the assay used for apoptosis detection \(^{(50)}\).

In the current study, we detected apoptotic process, using DNA content assay where DNA content increased with increasing cell division and decreased with increasing cell death. DNA content of homogenate liver samples obtained from CCl\(_4\) intoxicated rats was very highly significantly decreased at 72 hrs as respect to non-toxicated group. Our results agree with Shi et al.\(^{(58)}\), how demonstrated that apoptosis in hepatocytes was found as early feature of intoxicated liver injury.

This work was designed to assess the parallelism between immunological, biochemical and histochemical studies. Therefore, we determined the prevalence of (NOSA) in hepatotoxic rat groups induced by CCl\(_4\). The titers of (NOSA) were assessed by indirect immunofluorescence (IFL). In addition, the possible reaction between serum alloantibodies and isolated splenic lymphocytes of the rat were also examined.

The present study demonstrates that there is no prevalence of (NOSA) in the serum of intoxicated rats and there is no significant change between intoxicated and non-toxicated rats in the titer of autoantibodies (titer ≤ 1:40). This observation indicates that CCl\(_4\) has no immunogenic effect on intoxicated 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.\(^{(51,52)}\) who reported that experimental autoimmune hepatitis could not be induced in Lewis rats. In addition to the previous Smialowicz et al.\(^{(53)}\) demonstrated that hepatotoxic agents is not immunotoxic in the rats at dosages that produce overt hepatotoxicity. Furthermore, there was no difference in the antibody response to sheep red blood cells in another set of rats dosed at 40,80or 160 mg/kg/day CCl\(_4\).

The obtained results described immunoreactivity of expected induced autoantibodies with allo-antigen (splenic lymphocytes) of the same rat at different time's intervals, using in vitro lymphocytoxicity assay. These results indicate that no in vitro reactivity occurred between serum autoantibodies and isolated splenic lymphocytes in both CCl\(_4\)-intoxicated and non-toxicated groups over 12 weeks. These observations indicate the absence of hepatitis autoantibodies from the serum of intoxicated rat groups.

Interestingly, the current results showed that CCl\(_4\) molecule failed to stimulate immune system of rats to secrete autoantibodies. This result is in agreement with the results of Bahia-Oliveira et al.\(^{(54)}\) and Hirsch et al.\(^{(55)}\) who reported that nonimmunized rats did not develop significant levels of hepatitis autoantibodies.

The histological results showed that the normal structure of lobules was destroyed and pseudolobules formed, steatosis and inflammation were noted after 24 hrs of CCl\(_4\)-induced liver injury but fibrosis resulted at 72 hrs and complete cirrhosis did not occurred over 12 weeks in rats exposed to CCl\(_4\). These results are differ than obtained by Weng et al.\(^{(46)}\) who found that hepatic fibrosis stage was obtained at the fourth week after intraperitoneal injection of CCl\(_4\) (dissolved in olive oil with a proportion of 4:6) at 0.3 mL/kg of body weight, for 2 consecutive days a week for 16 weeks.

In the current study, a significant positive correlation was found between fibrosis index and fibrosis score in CCl\(_4\) (r= 0.90). This good 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 new fibrosis index biomarker may be used as a useful biomarker in the diagnosis and follow up liver diseases patients.

In conclusion, we showed that a significant positive correlation and association of hepatic concentration of hydroxyproline, activity of collagenase and (ALT and AST released from hepatocytes) with the severe hepatocyte necrosis, inflammation and fibrosis induced by CCl\(_4\) toxicity. Moreover the activity of antioxidant enzymes (SOD, catalase and GSH content), platelets count and DNA content are correlated inversely with hepatic fibrogenesis in intoxicated rats \(^{(59)}\). Cytological accumulation of T-lymphocytes in the tissue sections was observed at times paralleled with the observation of biochemical and histochemical markers. Finally, a parallelism study was demonstrated between biochemical, immunological and histochemical parameters of liver injury induced rats by CCl\(_4\).

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