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## RESEARCH ARTICLE

### Assessment of Hepatic Fibrosis by Image Analysis Software in relation to Physiological Markers.

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#### Abstract

**Background:** The chronic liver disease is one of the most deadly diseases in Egypt and around the world; perfect diagnosis methods are urgently needed. The proper assessment of liver fibrosis offers acute information for both perfect diagnosis and accurate therapeutic decision-making. Although digital image analysis (DIA) is a promising method for quantitative assessment of liver fibrosis, it has not been fully evaluated in practice yet. The present study was designed to explore the possibilities of using open access image analysis software (ImageJ) for accurate quantitative assessment of liver fibrosis.

**Methods:** This study investigated the use of digital images analysis by software ImageJ of liver sections stained by sirius red (SR). Reproducibility of this technique was tested in comparison with the a semi-quantitative score of liver fibrosis as well as other markers of fibrosis including physiological markers of liver functions such as alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT). In addition, it was tested compared to serum marker collagen IV (Col IV) and hepatic hydroxyproline. **Results:** From physiological point of view; hepatic hydroxyproline, serum Col IV and ALT showed higher accuracy, sensitivity and specificity than other physiological markers with area under curve (AUC) of 0.935, 0.955 and 0.902 respectively. On the other hand, DIA of SR by ImageJ software had shown the best accuracy with AUC of 1.0.

**In conclusions:** our novel data prove that the use of ImageJ with semiautomatic color segmentation is a reliable and practical way of measuring collagen proportionate area.

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#### Introduction:-

Liver fibrosis is known as the formation of too much quantity of extracellular matrix, forming scar tissue, in the liver parenchyma (1). Advanced liver fibrosis may lead to cirrhosis, liver failure, and portal hypertension and often end up with liver transplantation (2). Therefore, the accurate quantification of fibrous tissue in liver sections is essential for both investigational and clinical studies. Several studies have attempted to categorize serum markers that associate with the amount of fibrosis and thus could be used, with feasibility, with liver biopsy or could substitute it (3). Currently, semi-quantitative scoring systems can be used to evaluate the degree of fibrosis (4-6). Such scoring systems are subjective, requiring an experienced pathologist exhibiting significant inter- and intra-observer variation (7). In addition, it lacks the accurate evaluation of fine morphological details that could be done by a purely quantitative technique. As a result, recent studies have used computerized image analysis to assess hepatic fibrosis to overcome this problem. These quantitating methods aimed to be more accurate than, those determined by semi-quantitative scoring methods, without requiring the presence of an experienced pathologist (8).

The current study was designed to explore the possibilities of using open access image analysis software for quantitative assessment of CPA in liver tissue sections in correlation to physiological fibrotic markers and a semi-quantitative scoring system of liver fibrosis induced experimentally using carbon tetrachloride (CCl<sub>4</sub>). In addition, an open-access software for image analysis was used for quantitative assessment of CPA in liver tissue sections stained with SR.

## **Materials and Methods:-**

### **Chemicals:-**

Carbon tetrachloride was purchased from lobachemie (Mumbai, India). Chloramine-T, p-dimethylaminobenzaldehyde (Ehrlich's reagent), sodium acetate, citric acid, trisodium citrate, perchloric acid, isopropanol, sodium hydroxide, and acetic acid were purchased from Fisher scientific (St. Louis, USA) and trans-4-hydroxy-L-proline were purchased from Sigma-Aldrich (St. Louis, USA).

### **Animals:-**

Adult male Wistar rats (150-180 g) were given free access to tap water and food. All animals received humane care in compliance with the NIH and the Research Ethics Committee Criteria for Care of Laboratory Animals at Nile Center for Experimental Research, Mansoura, Egypt.

### **Experimental design :-**

In this experimental study, forty eight male Wistar rats were divided into 6 equal groups (each 8 rats) as follow; 1) Normal control group: served as normal healthy rats that did not receive any treatment, 2) Vehicle-treated group: these were treated with olive oil, 3) CCl<sub>4</sub> (2W) group: received CCl<sub>4</sub> intraperitoneally (0.5 ml/kg) twice a week in olive oil for 2 weeks (9), 4) CCl<sub>4</sub> (4W) group: received CCl<sub>4</sub> in olive oil for 4 weeks, 5) CCl<sub>4</sub> (6W) group: received CCl<sub>4</sub> in olive oil for 6 weeks and 6) CCl<sub>4</sub> (8W) group: received CCl<sub>4</sub> in olive oil for 8 weeks. At the end of the experimental period for each group, rats were anaesthetized by diethyl ether and sacrificed. Blood samples of each rat were withdrawn in test tubes. The tubes of blood were left for coagulation then centrifuged at 3000 rpm for 15 minutes to obtain serum for further biochemical analysis. The abdomen was opened through a midline laparotomy and the liver was rapidly removed, washed with normal saline. Part of the liver was placed in 10% formalin for histopathological examination and the other part was stored in liquid nitrogen (-196°C) for further analysis of hydroxyproline content in liver homogenates.

### **Biochemical markers of liver injury:-**

The serum activity of ALT, AST, ALP and GGT was determined using a commercial kit in accordance with manufacturers' instructions (ELITech Group, Paris, France). The serum activity of Col IV was determined using a commercial kit in accordance with manufacturers' instructions (Usen Life Science Technology, Wuhan, China).

### **Measurement of hepatic 4-hydroxyproline content:-**

Hepatic 4-hydroxyproline content was quantitated colorimetrically using the chloramine T method as previously described by Lee, Shun (10) with minor modifications (11). In brief, liver specimens (50 mg) were weighed in Eppendorf tubes and hydrolyzed in 0.5 ml of 2 N NaOH at 100° C for 2 hr. The hydrolysate was then cooled and centrifuged at 8000g for 10 min.

Chloramine T solution was prepared by dissolving 1 part of 7% chloramine T to 4 parts of citrate/acetate buffer (pH 6.0, 57 g sodium acetate, 37.5 g trisodium citrate, 5.5 g citric acid, 385 ml of isopropanol, and dissolved in H<sub>2</sub>O to a final volume of 1 L). Two hundred microliters of the supernatant was added to 0.125 ml of the prepared chloramine T solution and then was incubated at room temperature for 10 min. Thereafter, 0.75 ml of Ehrlich's solution was added. The Ehrlich's solution was prepared by dissolving 2 g of P-dimethylamino-benzaldehyde in 3 ml of 60% HClO<sub>4</sub> and then mixed with 9 ml of isopropanol. The final mixture was incubated at 60 °C for 35 min, then at room temperature for 10 min. The mixture absorbance was determined at 560 nm. Standard solutions containing authentic 4-hydroxy-L-proline were treated likewise. The standard curve was linear in this range (r = 0.99). The value of hepatic 4-hydroxyproline content was expressed as µg/g wet tissue.

### **Hepatic histopathological evaluations:-**

Portions of livers were fixed in 10% neutral buffered formalin solutions for 24 hr. Standard histopathological techniques were followed for processing the tissue and preparation of paraffin blocks. Fibrosis and CPA were evaluated in sections (5 µm thick) stained with SR using the score of Zhao et al, with modification (12). The

following scores were used to quantify fibrosis: F0 – no fibrosis; (F1) Central only in some lobules; (F2) Central only in most of the lobules; (F3) Central with short septa without bridging; (F4) Central fibrosis with bridging central –central; (F5) formation of cirrhotic nodules by fibrous septa which are delineating the portal lobule; (F6) Focal sub-segmentation of the portal lobules; (F7) Diffuse sub-segmentation of portal lobule; (F8) small separate cirrhotic islets separated by wide fibrous septa.

#### **Image acquisition :-**

All slides were examined by a light microscope Olympus model Cx31rtsf (Tokyo, Japan) and image acquisition was done by digital camera (Olympus Epl1, Japan) at a resolution of 4032×3024 pixels = 12192768 attached to a light microscope (Olympus Cx31rtsf) connected to a compatible personal computer Intel Core i3-2350M (2.3 Ghz, 4 Gb memory PC running on Microsoft windows 7). For each tissue type, a number of 5 images of random low-power fields (x40)/specimen of liver were saved from the digital slides in JPG (Joint Photographic Experts Group) format. The fibrosis ratio was the total area of fibrosis (A<sub>fib</sub>) divided by the total area of the section.

#### **Image processing:-**

Image analysis methods were performed using ImageJ software; version 1.48d (developed by Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) (13) and Java (64-bit) engine. This software was chosen since it is freely available and is platform independent.

### **Results:-**

#### **1. Physiological Markers:-**

Mean values of serum markers activity of liver functions including, ALT, AST, ALP, GGT, are shown in figure 1. Insignificant differences were demonstrated between normal and vehicle-treated groups ( $p < 0.001$ ). Treatment with CCl<sub>4</sub> induced a significant dose-dependent increase in the activity of serum ALT, AST, ALP and GGT and in the progressing of fibrosis stage reaching a maximum at the eighth week.

Mean values of serum Col IV and hydroxyproline content in liver tissues are shown in figures 2. They showed insignificant difference between normal and vehicle groups ( $P \leq 0.001$ ). Moreover, the current data demonstrated gradual increase in the serum Col IV and hydroxyproline content with increasing the dose of CCl<sub>4</sub> that corresponds to fibrosis progression. In order to identify the most accurate physiological marker, data was analyzed by means of the ROC curve, as shown in Table 1.

#### **Description of DIA method**

##### **a. Image phases:-**

After launching ImageJ picture editor and processing tool, the desired microscopic digital image is opened either by **File > Open** or by simply dragging the image to ImageJ's toolbar. Image segmentation of RGB image into different channels is based on the split channels parameter using the command **Image> color> split channels**. The simplest way of segmentation is based on the difference in the intensities of objects and background. This resulted in splitting the image into three 8-bit grayscale images containing the red, green and blue components of the original are performed. The green channel provided the best separation of these regions to isolate collagen.

To fix an uneven background the image was adjusted based on subtracting background parameter using the command **Process > subtract background**. The rolling radius used was 100, only the light background option has been checked (figure 3). Then a binary mask that contained only two values of the resulted subtracted image was done using **process > binary > make binary**. The first value signified that the pixel belonged to the background and the second signified that a pixel belonged to the object. To reverse black and white regions Edit>invert as it involves flipping' the intensities: making the higher values lower, and the lower values higher. To reverse black and white regions the command **Edit>invert** was used as it involves flipping' the intensities. After that the command **Edit>Selection>Create Selection** was used to create a selection from the inverted image so that fibrotic areas was identified from the background. It represented the borders of the objects we were interested in (figure 4).

Finally, before doing measurement the parameter to be measured, area and Limit to threshold, was set using **Analyze > Set Measurements** (figure 5). Measurements chosen were performed using the command **Analyze > measure**. ImageJ displays the results in a separate window (Results) that may be saved in one of the popular data storage file formats (e.g. csv or xls). This data is then available for further statistical analysis and data mining.

#### b. Creating Macros for Sirius Red :-

While performing all steps mentioned before on phase two for SR stain, they were automatically recorded on the opening window using the command Plugins > Macros > Record. The recorded macro was saved in the plugin folder by using the editor's File>Save As command. Macrocode is stored in text files (.txt and .ijm extensions). ImageJ automatically installed the macros when it started up which was follow: run("Split Channels"); run("Close"); run("Subtract Background...", "rolling=100 light"); set Option("Black Background", true); run("Make Binary"); run("Invert"); run("Create Selection"); run("Set Measurements...", "area limit display redirect=None decimal=9"); run("Measure");.

#### c. Statistical analysis for DIA of SR

Mean values of CPA by ImageJ in liver sections stained with SR is shown in figures 6. CPA determined by ImageJ showed significant elevation in CCl<sub>4</sub> treated groups compared to normal control and vehicle -treated groups (p < 0.001). Also, strong correlation was found between serum marker ALT, AST, ALP, GGT, col IV and hydroxyproline and DIA of SR (Figure 7). Moreover, the AUC for DIA of SR was 1.00 at cut off value 1.35. In addition, the sensitivity for DIA of SR was 100% while the specificity was 100%.

#### d. Statistical analysis for fibrosis score

Mean values of histopathological scoring examination of fibrosis in the liver sections stained with SR are shown in figure 8 and Table 2. Fibrosis scoring index of SR stain showed significant elevation in CCl<sub>4</sub> treated groups compared to normal control and vehicle-treated groups (p < 0.001). In addition, there was positive correlation between fibrosis score index and DIA of SR (Figure 9).

### Discussion:-

Liver biopsy represents the gold standard for damage evaluation, but noninvasive serum markers that show liver fibrosis progression are actual goals both in adults and especially in children. Carbon tetrachloride (CCl<sub>4</sub>) is one of the most commonly used hepatotoxins in the experimental study of liver diseases. Many studies have shown that CCl<sub>4</sub> administration in the rat increased the serum activities of ALT, AST, ALP and GGT when compared to control (14-19). This was confirmed in the current study in which measuring serum activity of ALT and GGT was more specific than AST and ALP for fibrosis according to their AUC values.

These parameters does give information about important aspects of liver function however, they do not assess severity of liver fibrosis or cirrhosis (20). In the study of Lu, Zeng (21) the activities of ALT, AST, ALP and GGT were all related to the staging of fibrosis. In a study of 456 patients with Chronic Hepatitis B (CHB), AST and ALT showed a significant correlation with advanced fibrosis (22). In another study by Huang et al (2013) (32), it correlated eight serum markers (GGT, HA,  $\alpha$ -macroglobulin, ALT, AST, platelet count, prothrombin time and albumin) with CPA of SR and masson trichrome. This is with agreement with the present study where ALT, AST, ALP and GGT activity show a positive correlation with values of image analysis of liver sections stained SR.

Type IV collagen is one of the most abundant proteins in the ECM that was investigated as a surrogate marker of liver fibrosis (23). Many studies have shown that administration of CCL<sub>4</sub> could cause increase in concentration of serum Col IV (24-26). This was in agreement with the findings of the present study, which demonstrated gradual increase in the serum type Col IV with advancing stage of fibrosis. The accuracy, specificity and sensitivity for serum Col IV were 0.955, 96% and 87% respectively. In addition, a positive correlation was demonstrated with the CPA measured by ImageJ in SR stained liver tissue. These findings are in agreement with Murawaki, Ikuta (27) who reported that the serum Col IV level was better for differentiating moderate or severe fibrosis from no or mild fibrosis in HCV with AUC = 75%. Also, same findings were demonstrated by Li, Piao (29). It demonstrated that serum Col IV increased significantly in hepatic fibrosis (dimethyl nitrosamine model) at week 1,2,3,4 and a positive correlation was recorded between the CPA measured by CMIAS image analysis system in SR stained tissue and serum levels of Col IV. These findings suggest that measurement of serum Col IV could be a reasonable index for assessment of liver fibrosis.

The alteration of hydroxyproline levels in the liver is considered as an index of collagen metabolism and give useful information about the biochemical and pathologic events of hepatic fibrosis (30). A gradual and progressive increase in the hydroxyproline content in the liver tissues significantly demonstrated as the CCL<sub>4</sub> dose increase and fibrosis became more advanced. In addition, a significant positive correlation is observed between digital image analysis (DIA) of SR and hydroxyproline levels in the corresponding liver tissues. Matching findings were demonstrated by Nakayashi, Takamatsu (31) indicating a highly significant correlation between the biochemically measured

hydroxyproline content of liver samples and the CPA of double stain of SR and Fast green SF stain. Also, hydroxyproline showed a significant positive correlation with the staging scores proposed by Zhao, Wang (12).

Little universally acceptable criteria exist for animal models, (12) scoring system was used to assess the severity of fibrosis in CCl<sub>4</sub> and dimethyl nitrosamine models in rats. This seven-stage criterion has its own pathological features that can be distinguished morphologically. The degree of fibrosis in this study is recorded on the basis of the Zhao et al scoring system for staging of fibrosis in CCl<sub>4</sub>-induced liver fibrosis in rats with some modifications. Fibrosis scoring index, in the current study, showed significant elevation in the all CCl<sub>4</sub>-treated groups compared with the control value ( $p < 0.001$ ). It had shown an AUC of 1 with 100% and 100% sensitivity and specificity, respectively. In addition, there was a significant positive correlation was demonstrated between the staging scores and increased relative fibrous areas in liver tissue for SR ( $r = .918, P < 0.001$ ). These findings are in agreement with that reported by Zhao et al. (12) in which a significant positive correlation was demonstrated between the staging scores and increased relative fibrous areas in liver tissue specimens in the rat model ( $r = 0.919, P < 0.001$ ).

Digital morphometry, in contrast to other morphologic methods, use personal computers and specific software, to perform precise and highly reproducible results and provide results in mathematical format (28). Several studies have attempted to correlate the image analysis measurements of fibrosis with the categorical stage scores. In a study by Huang, de Boer, SR stain could detect delicate fibrous septa better than trichrome. Both CPA of SR and CPA of Masson Trichrome correlated well with Metavir stage, whereas CPA of SR had better ability to detect cirrhosis with AUC of 0.95(33).

Recently, in the study of Huang, de Boer (34) to predict adverse clinical outcomes in CHC patients with all stages of fibrosis, CPA in SR stained liver tissue measured by Aperio Image Scope software showed a significant correlation between CPA and metavir stage. It also showed an excellent predictive ability for hepatocellular carcinoma development with an AUC of 0.92, which was significantly higher than that of metavir stage. This was in agreement with the present study where CPA of SR had excellent predictive ability for liver fibrosis detection with AUC of 1.00.

The present study presented a practical approach to perform image analysis of SR stained liver tissue. ImageJ software was chosen because is freely available along with extensive documentation. Additionally, the current study presented the required steps in order to perform the method. Moreover, it is possible to turn the manual method to automated one by recording it as a macro. The present findings confirm the validity of using ImageJ over the routine histopathological examination by pathologist. The examination is easy so that a technician can perform it.

From physiological point of view, hydroxyproline, Col IV and ALT showed the higher accuracy, sensitivity and specificity so it can be used for liver fibrosis diagnosis. In addition to being substantially less invasive, with no risk of complications, eliminate sampling errors and observer variability. Moreover, measurements may be performed repeatedly and used in monitoring of fibrosis. However, as none are liver specific their results can be affected by comorbid conditions, requiring critical review for false positive and false negative results (31).

Figure legends:-

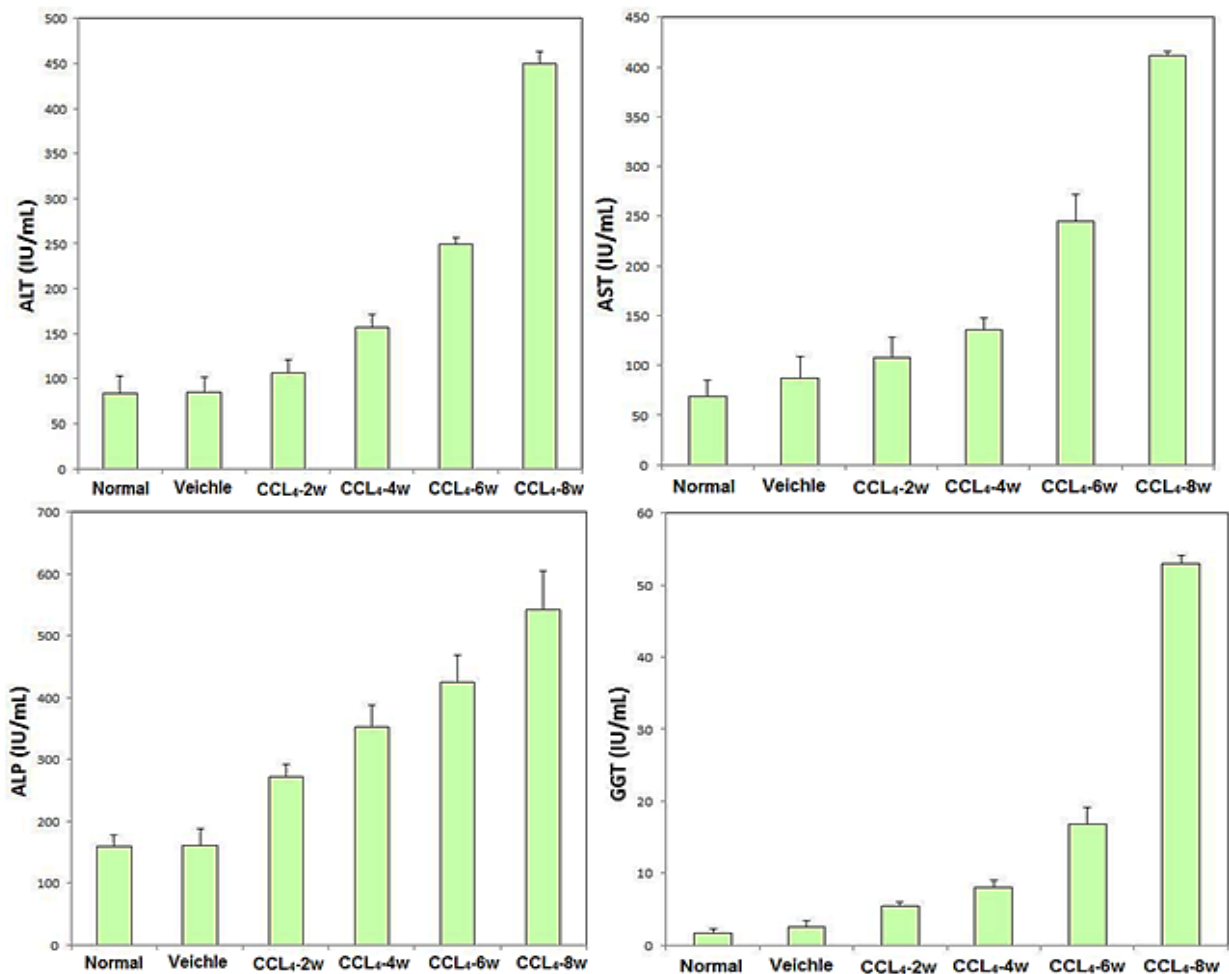


Figure 1: Serum activities of different physiological marker in the control and CCl<sub>4</sub>-treated groups

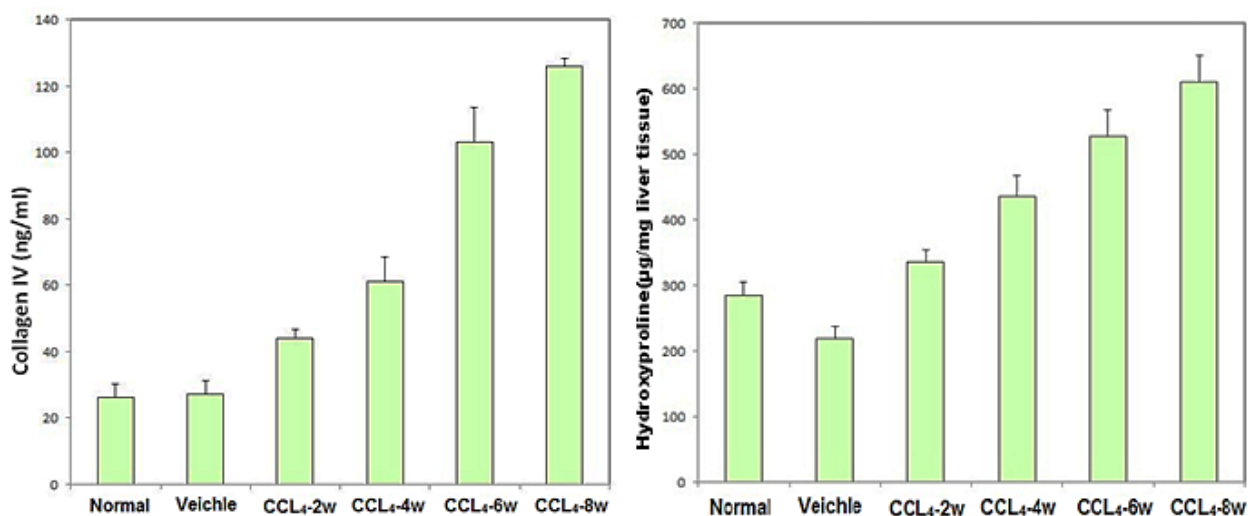


Figure 2: Serum col IV and liver Hydroxyproline content in the control and CCl<sub>4</sub>-treated groups

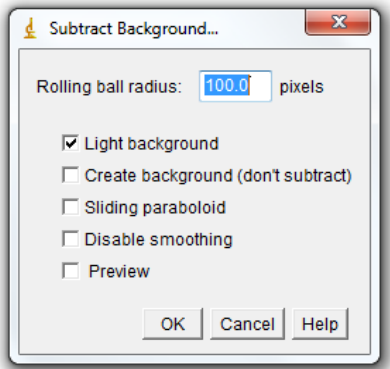


Figure 3: ImageJ's Analyze subtracting background setting panel

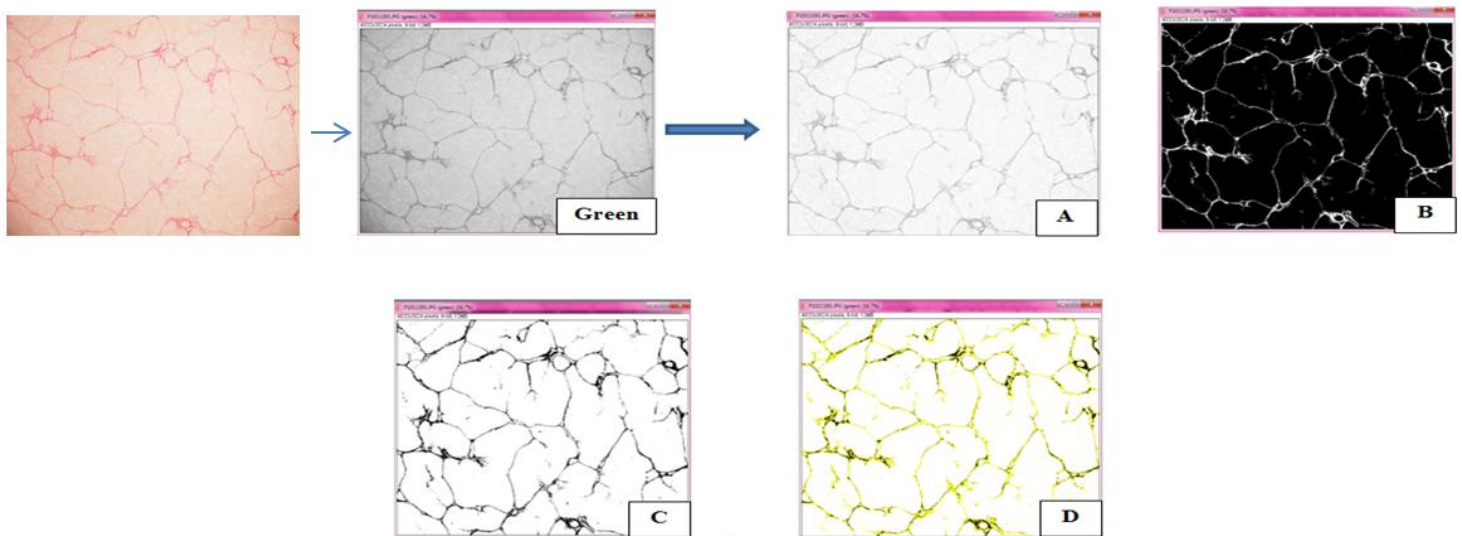


Figure 4: By choosing only the green channel to complete the DIA for the original image, Different steps of image processing starting from subtracting the background (A), converting the image to a binary image (B), reversing black and white regions (C) and finally creating a selection from the inverted image so that fibrotic areas can be identified from the background (D).

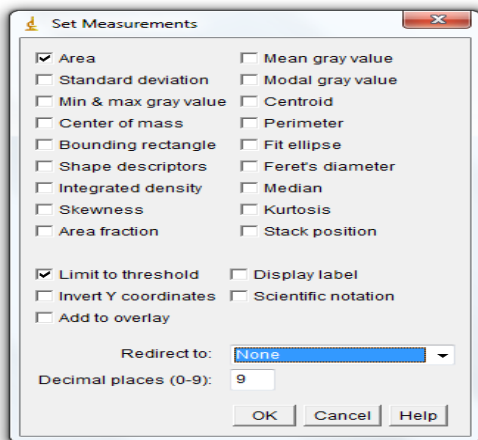
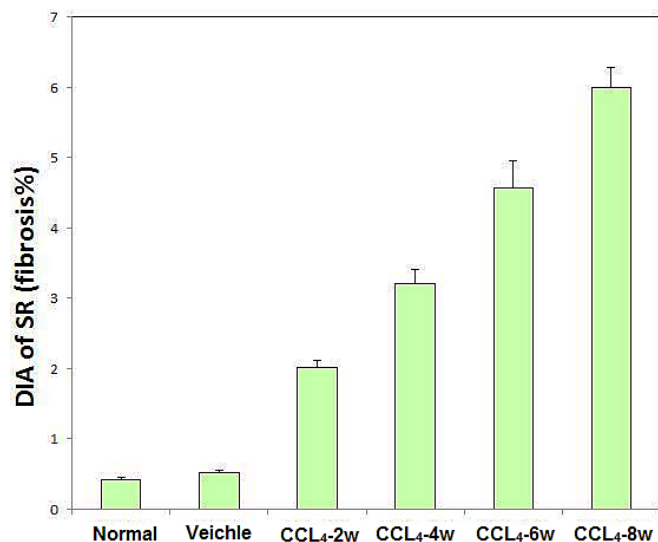
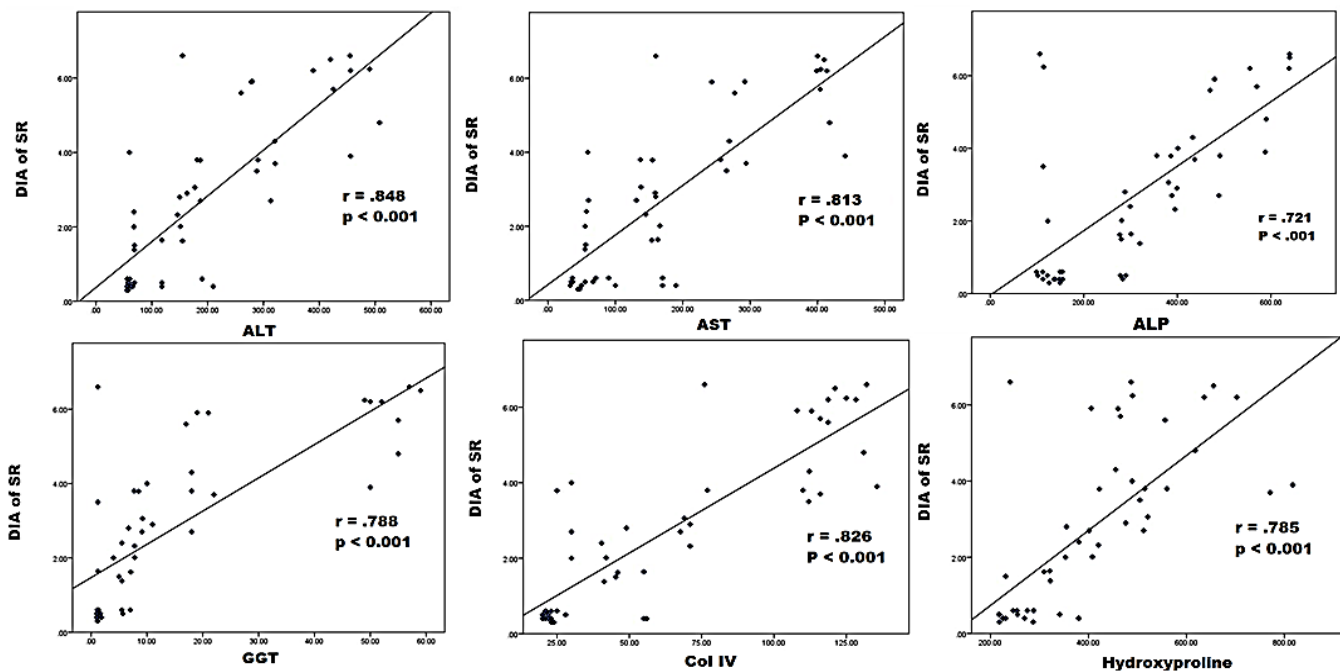


Figure 5: ImageJ's Set Measurements settings panel



**Figure 6:** Mean values of CPA by image analysis by ImageJ software for liver sections stained with SR in in the control and CCl<sub>4</sub> treated groups.



**Figure 7:** Correlations between different physiological marker and image analysis by SR



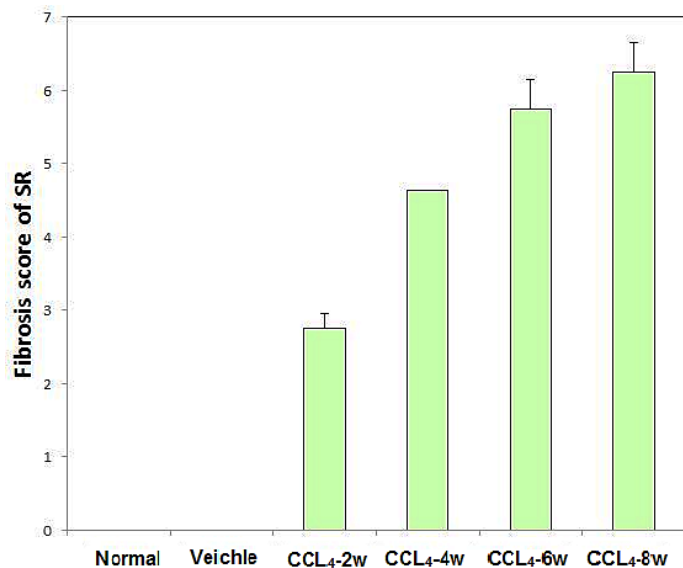


Figure 8: Fibrosis score index of SR in the control and CCL<sub>4</sub> treated groups

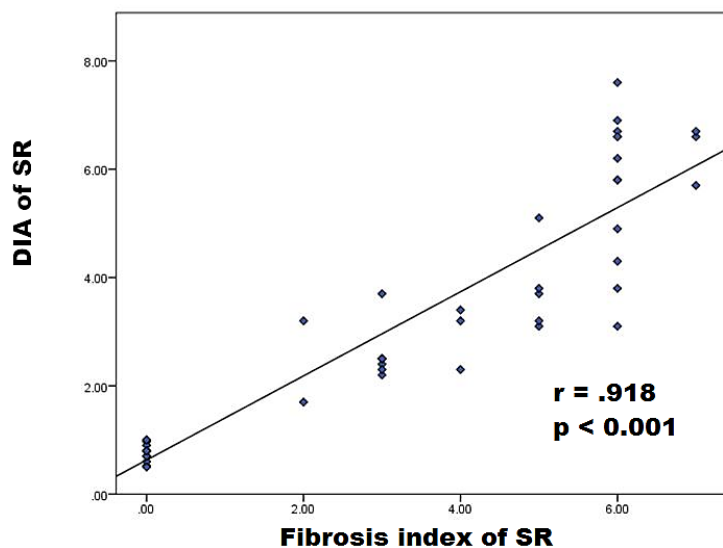


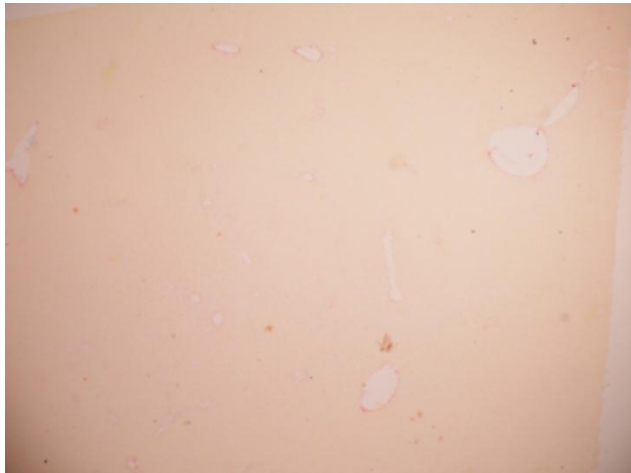
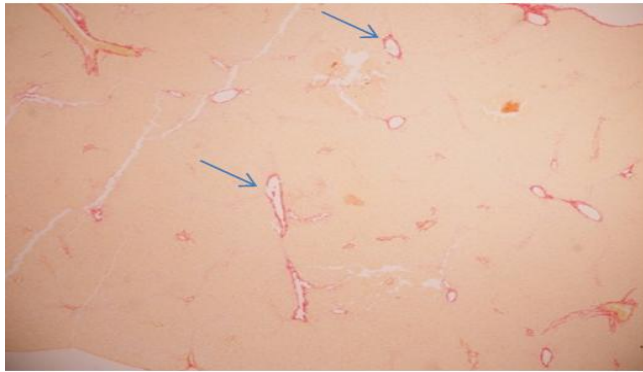

Figure 9: Correlations between pathological fibrosis score and image analysis

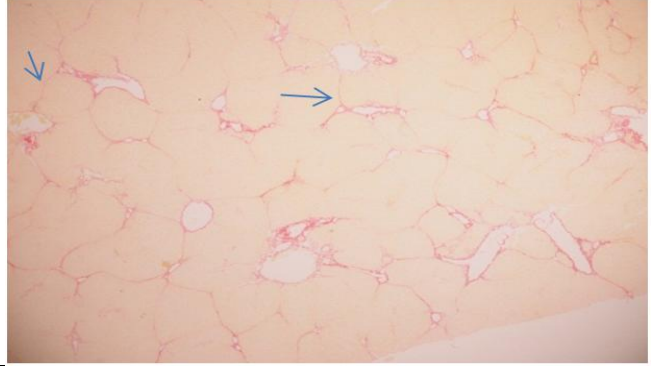
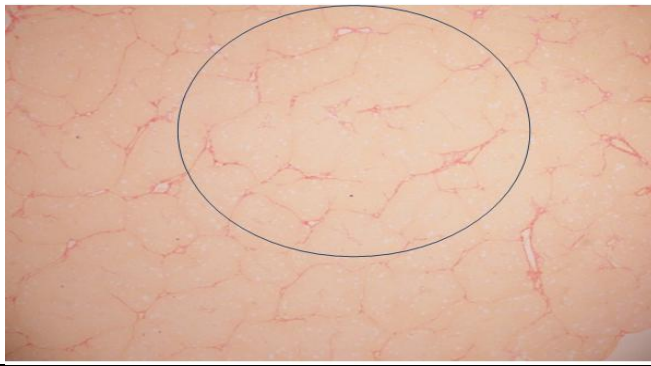
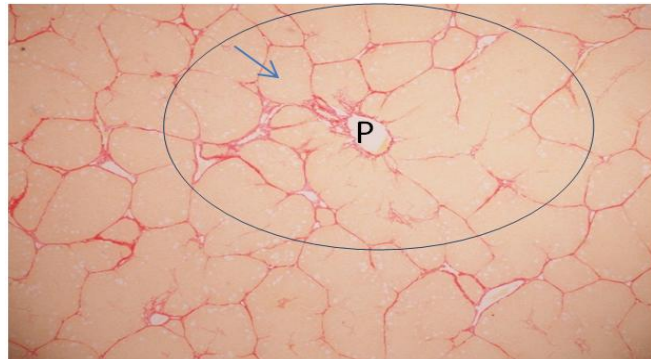
Table legends:-

Table 1: AUROCs for different physiological markers of fibrosis.

Marker	Cut off	Sensitivity	Specificity	AUC
ALT	132	81.3%	87.5%	0.902
AST	115.5	81.3%	81.2%	0.852
ALP	279	84.4%	87.5%	0.888
GGT	2.95	90.6%	81.2%	0.930
Col IV	29	96.9%	87.5%	0.955
Hydroxyproline	298.8	93.8%	81.2%	0.935

Table2: Fibrosis score index

Appearance	Score	I.A
	<p><b>F0</b> normal control group where central veins are very minimally encircled by fibrous tissues with no septa nor bridges</p>	.36%
	<p><b>F2</b> pericentral fibrosis is located in most of the central veins</p>	2.1%
	<p><b>F3</b> short septa of pericentral fibrosis extend from the central vein but not connected to other central veins</p>	2.4%

	<p><b>F4</b></p> <p><b>Central-central bridging by fibrosis septa</b></p>	<p><b>4.1%</b></p>
	<p><b>F5</b></p> <p><b>septa join central veins to form nodules corresponding to the portal lobules</b></p>	<p><b>4.3%</b></p>
	<p><b>F6</b></p> <p><b>fibrous septa form nodules corresponding to the portal lobules with focal sub segmentations</b></p>	<p><b>5.3%</b></p>

### Conclusion:-

In conclusion, morphometric assessment of CPA is accurate and reproducible method for evaluating fibrosis in liver biopsies. There is a significant correlation between the morphometric image analysis of the percentage area of fibrosis and the staging system of fibrosis in liver, serum Col IV as well as hydroxyproline content in liver and liver function enzymes (ALT, AST, ALP and GGT).

### Abbreviation:-

ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate aminotransferase; AUC: area under curve  
 CCl<sub>4</sub>: Carbon Tetrachloride; Col IV: collagen IV; DIA: Digital image analysis; GGT: gamma-glutamyl transferase;  
 SR: sirius red.

### Competing Interests:-

The authors declare that they have no competing interests.

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