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A Quantitative Description of Lipid and Extracellular Matrix Proteinaceous Fibers in Hepatic Fibrosis of a Rat Model by *ImageJ* using Nano-Images

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Abstract

We made a histological and ultrastructural studies using *ImageJ* freeware and Origin pro statistical analysis of the site of lipid accumulations and ECM pertinacious collagen fibers in a rat model of hepatic fibrosis using CCl_4 and ethanol dual effect and using nano GTE as the main factor to diminishes both hepatic lipid and ECM fibers. This imageJ observation indicates and re-enforces the notion previously published by the same author that Nano GTE is a strong therapeutic agent to work against the oxidative effects of chemicals like CCl_4 and ethanol.

Keywords: Lipid; Fibber; Chitosan nano-green tea; Bio-nano particle; Hepatocytes; ImageJ

Introduction

It is now recognized that in the hepatofibrotic liver, lipids and fibrous proteins are known as steatosis and fibrosis respectively are enormous in liver parenchyma [1-4]. In several of our recently published works, although we have managed to establish their presence as an outcome of liver fibrosis and how nanogreen tea extract managed to remediate it in several technics [4,5] yet the analysis of their features in tissues often remains qualitative. Thus, quantitative microscopy technique for imaging localized lipid and ECM fibers environments and measuring them in fixed tissues is needed to be explored.

This approach directly examines the organization of lipid and fibers. Image analysis tools have been developed to quantify such features, but they often involve an image processing stage that may bias the output and/or require specific software.

This paper aims at confirming once again what have been published earlier [5] about lipid accumulation and ECM fiber formation during the process of hepatic fibrosis by adopting combined ImageJ and Origin Pro 2017 for statistical Analysis [5-7]. These two Freeware combined for the first time - in analyzing nanoscopy images obtained from fibrotic group and group treated with nanogreen tea extract providing this new tool for exploring and re-enforcing the presence of the two district features that occurred during the various stages of hepatocirrhosis; lipid bodies and extracellular matrix fibers and how nanogreen tea extract remediates both [4].

Materials and Methods

Experiments

Preparation of chitosan nano-GTE: Preparation of green tea extract (GTE) and encapsulation of GTE within chitosan nanoparticles (including characterization methods) to make chitosan nano-GTE was done as described in our earlier paper [4].

Animals: Forty male Sprague-Dawley rats weighing between 200-250 gms were used in this study. All animals were obtained and housed in the Animal House, Department of Biological Sciences, Kuwait University. The Animal House is a well-established facility with an International Code of Ethics. The rats were kept in plastic cages in a controlled environment of 40% humidity at 22°C, with 12 hr light/dark period and treated gently. All rats had free access to tap water ad libitum and pellet rodents chow (SDS, Witham, UK). The composition of the diet was 14.7% protein, 2.6% fat, and 5.3% cellulose. Rats were divided into 4 groups (10 in each group) based on different oral treatments administered to them. Subcutaneous doses of 40% CCl_4 and oral doses of 25% ethanol, chitosan, and chitosan nano-GTE were administered to the animals as detailed in Table 1.

Tissue preparation for LM and SEM: Rats from each group were carefully anesthetized with ether followed by dissection of the abdominal cavity to expose the liver. The liver was immediately soaked with cold 2.5% glutaraldehyde/sodium cacodylate fixative, pH 7.2, kept at 0-4°C for 2 hours then changed to a fresh fixative and left overnight. Tissues were then transferred to sodium cacodylate/sucrose buffer, 3 times for 20 minutes each, then transferred to 1% OsO_4/PO_4 buffer for 2 hours and blocked in Epon.

Semi-thin sections were cut using Leica ultra-microtome for toluidine blue staining and photography.

For SEM, critical point drying (CPD) was used. The blocks were fixed on stubs and put in the sputter coater for shadowing the specimen with platinum/gold. Investigation and image acquisition were done on the Variable Pressure Field Emission remotely operable scanning electron microscope (Leo Supra 50VP) operated at 30 kV.

Computational image analysis

Hepatic lipids and collagen fibers were measured using ImageJ Freeware version V 1.51A plug-ins, downloaded from the NIH website (http://rsb.info.nih.gov/ij) were used (Scheme 1). ImageJ Freeware V 1.51A is a public domain, image processing program developed at

Label	Tissue Total Area	lipid Area (*Pixel*)	Area (%)
control (*Green*)	3117876	100749	3
eth+ccl_4 (*Green*)	3145720	768008	28
eth+ccl_4+gt (*Green*)	3145728	121917	20

Table 1: Quantification of the main tissue area and quantification of lipid and percentage.

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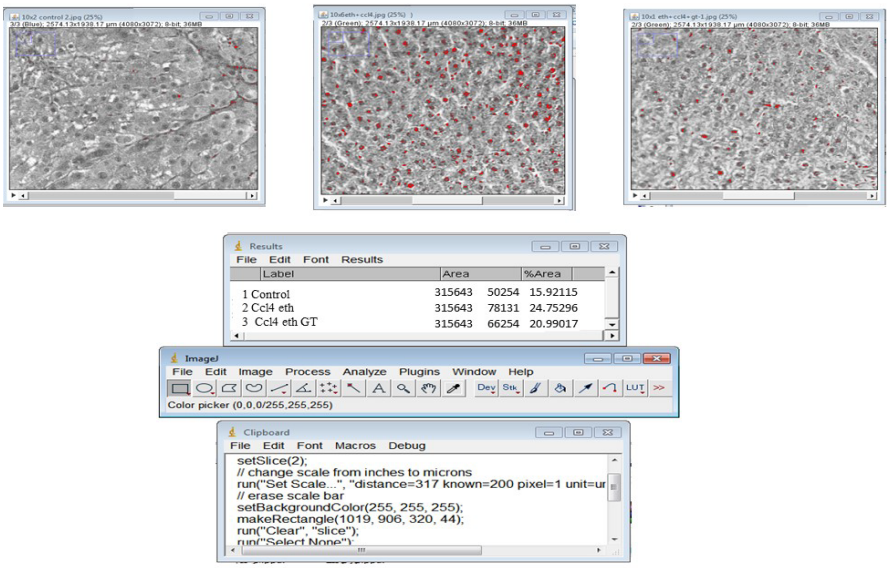

Scheme 1: Shortcut screen from imageJ software for Collagen fiber and macro program analysis.

the National Institutes of Health NIH. http://rsb.info.nih.gov/ij; It is a public domain Java image processing program inspired by NIH, USA [6-8].

Quantification of hepatic lipid content before and after fibrosis

Computational image analysis for the determination of the damaged regions of the tissue after the effect of CC4 and ethanol and the treatment with nanoGTE was carried out primarily with the this software package. Quantifications for the damaged, undamaged, and complete area of the tissue were carried out in pixels and the results were expressed in percentage of the ratio of the damaged to the complete tissue area [5]. To quantify the artificially made red-stained collagen in an image of a rat liver tissue section stained with H/E and/or Masson's Trichrome stains Table 2. The following four main steps have been followed:

I. Change the scale to Nanometer,

II. Convert the image to grayscale,

III. Segment (isolate) the red-stained collagen using thresholding,

IV. Measure the thresholded area.

We can fix this by measuring the length of the scale bar and using the Analyze>Set Scale command from the main menu program to set the scale to nanometers. To measure the scale bar, we used the straight line selection tool (a fifth tool from the left on the ImageJ toolbar) to create a line selection along the length of the scale bar as shown below in short-cut of the screen [6-8].

Open the analyze: Set Scale dialog box. Notice that the length of the line selection (317 pixels) is automatically entered as the "Distance in Pixels". Enter the scale bar length (200 µm) as the "Known Distance" and "um" as the "Unit of Length". ImageJ will automatically convert the "u" to the micrometer symbol (µ). Click "OK" and notice that the image size has changed to 858.95x646.74 µm [6,7].

Next use the image: >Type>RGB Stack command to split the image into red, green and blue channels. This is necessary because the Image>Adjust>Threshold tool only works with grayscale images. Try

Label	Tissue Total Area	lipid Area (*Pixel*)	Area (%)
control (*Green*)	315643	100749	15.9
eth+ccl$_4$ (*Green*)	315643	768008	44.7
eth+ccl$_4$+gt (*Green*)	315643	121917	21

Table 2: Quantification of the main tissue area and quantification of fiber and percentage.

to threshold an RGB image and you will get a lot beeps and the status bar message "RGB images cannot be thresholded". Move the slider to view each of the channels. Notice that the green channel has the best separation [7,8].

Use the Image>Stacks>Make Montage command to view all three channels at the same time: Select the RGB stack (with the Green channel selected) and press shift-t (Image>Adjust>Threshold). The "Threshold" tool opens and the green channel is automatically thresholded. Unfortunately, ImageJ is not able to correctly threshold this image so the threshold must be manually adjusted. Do this by moving the lower slider until the stained collagen is highlighted in red. Notice that the threshold level arrived at manually is about half the automatically set level of 175. Click on "Set" and set the upper level to 175/2, or 87. Setting a threshold that is a fraction of the automatically determined threshold will allow us to later create a macro that does not require manual thresholding [9].

Next we must erase the scale bar to prevent it from being included in the calculated area: To do this, use the rectangular selection tool to select the scale bar, then press Backspace (Delete on the Mac). If the current background is not white, use the color picker tool (Image>Color>Color Picker) to change it to white. Note that the current background color is indicated by the color of the border surrounding the eye dropper tool icon [7].

We are almost ready, but first we have to tell ImageJ what to measure by opening the Analyze>Set Measurements dialog and checking "Area", "Area Fraction", "Limit to Threshold" and "Display Label". Then press "m" (Analyse>Measure) and the area and percent area will be displayed in the "Results" window. Right click in the "Results" window and select

"Save As" to save the results as a tab-delimited text file or select "Copy" to copy the results to the Clipboard [6,8].

Another way to measure the red-stained collagen is to convert the thresholded portion of the image to a selection using Edit>Selecton>Create Selection, transfer the selection to the original RGB images by selecting it and pressing shift-e (Edit>Selecton>Restore Selection), and then pressing "m" (Analyse>Measure) to measure the area of the selection Table 3 [8].

Quantification of hepatic collagen content before and after fibrosis

Below is a macro that automates the analysis. To run it, copy it to the clipboard, switch to ImageJ, press shift-v (File>New>System Clipboard), click on the quot;Clipboard" window and press ctrl-r (Macros>Run Macro). Information about the ImageJ macro language is available at rsb.info.nih.gov/ij/developer/macro/macros.html [6-10]

Macro program

//select the green channel, which has the best contrast

run("RGB Stack");

setSlice(2);

//change scale from inches to microns

run("Set Scale...", "distance=317 known=200 pixel=1 unit=um");

Component	Control	CCl_4+ethanol	CCl_4+ethanol+GT
Lipid	3%	28%	20%
Fiber	15%	49%	22%

Table 3: Quantification Percentage of fiber and lipid in different group.

//erase scale bar

setBackgroundColor(255, 255, 255);

makeRectangle(1019, 906, 320, 44);

run("Clear", "slice");

run("Select None");

//set threshold

setAutoThreshold();

getThreshold(min, max)

setThreshold(0, max/2);

//measure area and area fraction

run("Set Measurements...", "area area_fraction limit display redirect=None

decimal=3");

run("Measure");

selectWindow("Results");

A second image is available for testing the macro. The & quot; Results & quot; window below contains the output resulting from running the macro on both images. The & quot; Label & quot; column displays the image title and channel [11].

Results

Lipid analysis

Once threshold was set at a proper scale, from normal control (Figure 1a), all lipid droplets in the damaged tissue under study due to

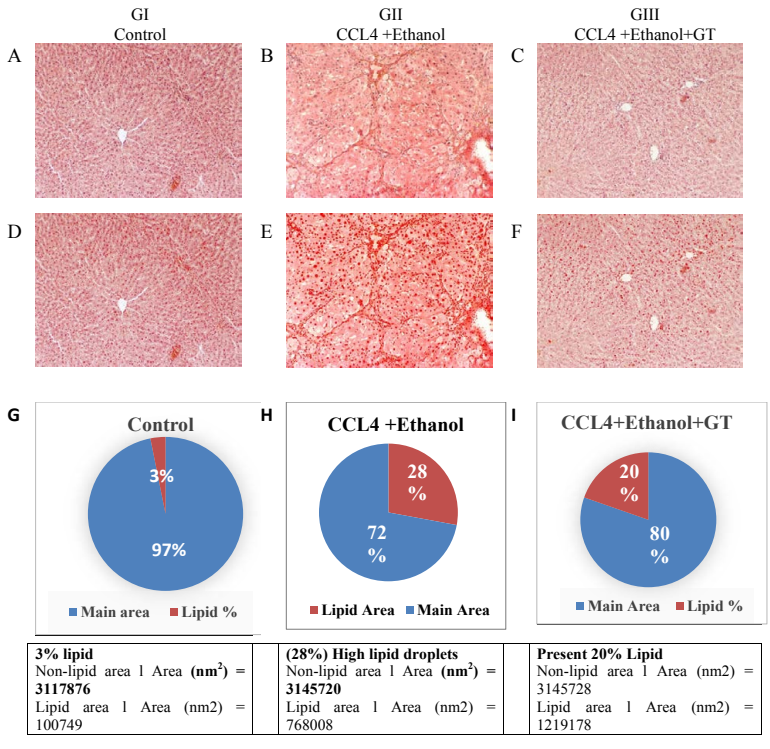

Figure 1: Quantification of lipid comparison with different group Protein fiber analysis.

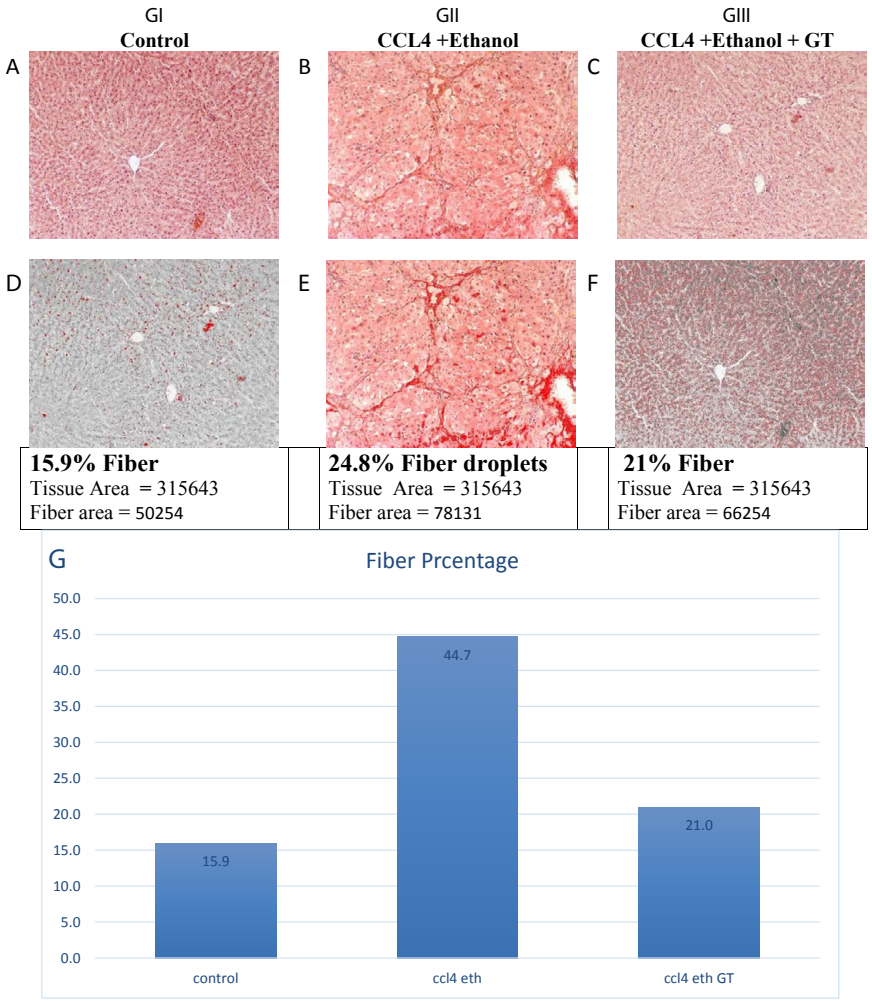

Figure 2: Quantification of Fiber comparison with different group.

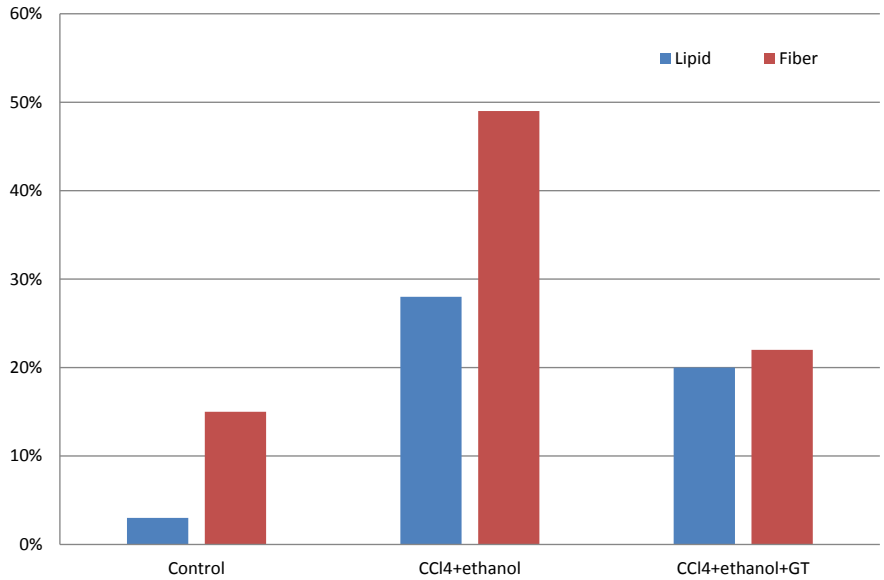

Figure 3: Quantification Percentage of fiber in different group.

the effect of CCl_4 and ethanol became visible bright red in color (Figure 1b). The total percentage of lipid was 28%. Comparing to the one treated with nanogreen tea extract in the presence of CCl_4 and ethanol 20% (Figure 1c). As indicated in the images of the control, the lipid in the damaged is was only 3% from the total non-lipid area l3117876 nm^2, while the lipid area was 100749 nm^2. In the completely damaged group, the total lipid was as high as 28%, where the non-lipid area was 3145720 nm^2 versus the lipid area 768008 nm^2. In the third group of ethanol/CCl_4 and nanoGTE, the total lipid are came down to 20%, where the non-lipid area was 3145728 nm^2 and the lipid area was 1219178 nm^2. [5,10].

Protein fiber analysis

Once threshold was set at a proper scale from normal control (Figure 2a), all fibers in the damaged tissue under study became visible bright red in colour (Figure 2b) with total percentage of fibers 49%. Comparing to the one treated with nanogreen tea extract in the presence of CCl_4 and ethanol 22% (Figure 2c). As indicated in the images of the control, the fiber in the damaged was only 15.9% from the total non-fiber area, In the completely damaged group the total fiber was as high as 44.7%. In the third group of ethanol/CCl_4 and nanoGTE the total fiber area came down to 21%, The two parameters for the three groups of rat are clear in the histogram where the big difference between the damaged case when ethanol/CCl_4 are used for three weeks to create fibrosis and the ethanol/CCl_4 and nanoGTE are used as antifibrotic therapeutic agent (Figure 2) [4]. The effect of nanogreen tea extract is clear in the tested group in comparison to the control group and the ethanol. CCl_4 group as shown in the two histograms provided (Figures 2 and 3). When nano SEM images were resolved and analyzed, It was found that there are two distinc types of fibers formed in the ECM during fibrosis stages; one due to the effect of CCl_4 (Figure 4A) while the other was due to ethanol (Figure 4B). A combined pattern of fibers were also discerned as in (Figure 4C). The ECM fibers were completely eliminated from the scene after three weeks of nanogreen tea treatment.

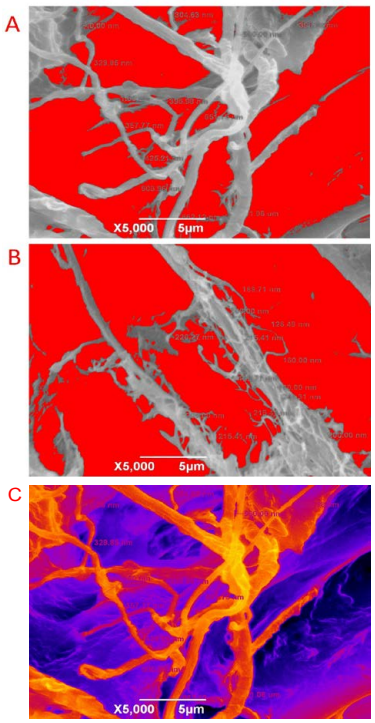

Figure 4: Showing fibrous proteins in the ECM of rat fibrotic hepatocyte with specified pattern. A. shows fibers formed due to the effect of CCl4, B. shows fibers formed due to the effect of ethano. C. shows image of combined fibers. X5000.

Discussion

Here we analyzed the previously published work on the effects of nanogreen tea extract on hepatic fibrosis using various histological and histochemical techniques and electron microscopy analyses; both TEM and SEM [11,12] by using the ImageJ software. This piece of work is aimed to confirm the therapeutic effects of nanogreen tea extract on hepatic fibrosis and how it reverses tissue structure and cell organelles to their normal norm and how it eradicates ECM fibers all over the place. This technique provides quantitative descriptions of the fiber arrays and their average orientation in ECM [13,14], directly from the histologic stained images on one hand and the nano images obtained from high-quality TEM and SEM images on the other. With these software combined, fibrillar structures, such as collagen, or the texture of various materials have been resolved quantitatively. The tool provides the location and quantification of both lipid droplets (steatosis) and the ECM fibrous proteins in the fibrotic liver before and after the treatment with nano-green tea extract and the average orientation and anisotropy of fiber arrays in a given region of interest [10,12].

As for Lipid Analysis, once the threshold was set at a proper scale, from normal control all lipid droplets in the damaged tissue under study due to the effect of CCl_4 and ethanol became visible bright red in color, with the total percentage of lipid was 28%. Comparing to the one treated with nanogreen tea extract in the presence of CCl_4 and ethanol 20% and with reference to the control group of zero damage. The total damaged are in control was found as 100749 and mean 153.00 while in CCl_4/ethanol group it was 314540 and mean 114.00 in CCl_4/ethanol/CCl4/ethanol/NanoGTE was 3145728 with mean 129.00 [5].

For protein fiber analysis; once the threshold was set at a proper scale, the damaged tissue under study became visible bright red in colour with the total percentage of fibers 49%. Comparing to the one treated with nanogreen tea extract in the presence of CCl_4 and ethanol 22% and the zero in control. The total damaged are in control was found as 15.9% and in CCl_4/ethanol group it was 44.7% and in CCl_4/ethanol/Nano GTE was 21% [5,9,12,15].

The two parameters for the three groups of the rat are clear in the histogram where apart from the control, it clearly shows the big difference between the damaged case when ethanol/CCl_4 are used for three weeks to create fibrosis and the ethanol/CCl_4 and nanoGTE are used as the antifibrotic therapeutic agent (5.11).

Once again imageJ technique confirms what was seen in previous work [11,12,16] that nanoGTE when used for rat hepatic fibrosis model treatment for a period of three weeks, will eradicate all ECM fibrous protein and most of the lipid in the cell cytoplasm almost to its normal norm [5]. When ECM fibers were resolved and examined with high quality SEM nanoimages,two distinct types of fibers were clearly visible; one was formed due to CCl4 and the other was formed due to ethanol. These fibers are native to the ECM of various tissues specifically accumulated when tiises are under abnormal or diseased condition [17,18].

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