Silymarin Versus Gold Nanoparticles efficacy in ameliorating CCl4- Induced Liver Fibrosis in Adult Male Albino Rats: A Histological and Immunohistochemical Study

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Abstract

Introduction: For patients with advanced liver cirrhosis, conventional therapies could not provide adequate improvement. Organ transplantation - in spite of its hazards - remains the only hope for them to avoid the risks of further progression to end stage. There is a demanding need for alternative therapy. Aim of the work: This study was performed to compare between the therapeutic potential of gold nanoparticles as a new line of therapy and silymarin in repair of liver fibrosis. Material and Methods: Forty-five male albino rats were equally divided into five groups; control group, CCl4- treated group, CCl4-recovery, silymarin-treated group and nanogold- treated group. By the end of the experiment, blood samples were collected for serological study (ALT and AST). Liver specimens for light and electron microscope examination were processed. Results: Histological examination of liver specimens showed that CCl4 caused variable pathological changes with elevated liver enzymes. These pathological changes were increased in CCl4-recovery group while, silymarin- and nanogold- treated groups showed improvement. The liver normal structure was regained in a better form in the nanogold group. Conclusion: CCl4 induced marked degenerative changes in the liver parenchyma and stroma of adult male albino rats. Administration of silymarin or gold nanoparticles improved the induced changes but gold nanoparticles were more effective.

Recommendation: Nanogold treatment could be advised in advanced cases of fibrosis not responding to medical treatment.

Key words: liver, fibrosis, histology, CCl4, silymarin, nanogold.

Introduction:

Liver fibrosis results from chronic damage to the liver. The main causes of liver fibrosis include chronic hepatitis C virus (HCV) infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH) (1). The Egyptian Demographic Health Survey (EDHS), a cross sectional survey including HCV biomarkers, was conducted in 2008 on a large nationally representative sample. It estimated HCV prevalence among the 15–59 years age group to be 14.7%. Accordingly, Egypt has the highest HCV prevalence in the world (2,3). HCV is one of the major causes of chronic liver diseases, which include
inflammation, fibrosis and cirrhosis. Furthermore, HCV has been associated with increased morbidity and mortality in hepatocellular carcinoma (4).

Experimentally, administration of carbon tetrachloride (CCl4) to rodents is a widely used model to study mechanisms of hepatic injury. CCl4 causes hepatocyte injury that is characterized by centrilobular necrosis that is followed by hepatic fibrosis (5).

Natural antioxidants such as phenols and flavonoids show protective actions in different models of toxin-induced oxidative stress. Silymarin is a mixture of flavonoid components, and this natural antioxidant is isolated from Silybum marianum (Milk thistle). Silymarin has anti-inflammatory, anti-oxidative and anti-carcinogenic activities (6-8). It is a powerful antioxidant used to ameliorate liver damage induced by various chemicals or toxins, including phenyl hydrazine, palmitate and carbon tetrachloride (9).

For patients with end-stage fibrosis (cirrhosis) or cancer, liver transplantation is the only effective treatment, but it is associated with several problems, including shortage of donors, operative damage, risk of immune rejection, and high costs. Also, liver transplantation induces several long-term side effects, such as chronic renal failure, post-transplantation lymph proliferative disorder, and cardiovascular complications. Therefore; there is a need for alternative therapies (10).

In complementary medicine, metal and oxide nanoparticles are currently being considered for a wide array of medical applications (11). Nano-sized particulates can be classified into two types; organic and inorganic. The most prominent inorganic nanoparticles are gold nanoparticles (AuNP), which can be rather easily modified in size, shape, and functionalization (12). Pure gold has been used for many decades. Besides its many beneficial effects, some toxic side effects have been reported to be associated with the use of gold compounds, but not pure gold (13). Several studies were focused on the application of gold nanoparticles in rheumatoid arthritis, liver fibrosis, diabetes mellitus and chronic myeloid leukemia (14).

Finding a new strategy for treating liver fibrosis and preventing progress to cirrhosis is of crucial importance in Egypt. The emerging field of nanogold therapy may provide a new hope for hepatic repair instead of the traditional treatment, liver transplantation. Using carbon tetrachloride induced liver fibrosis in adult male albino rats, the present study was conducted to compare between the therapeutic potential of gold nanoparticles as a new line of therapy for liver fibrosis and the effect of silymarin which is usually clinically used in hepatic repair in ameliorating liver fibrosis.

Materials & Methods

Forty-five male albino rats weighing 180–200 g were housed in a temperature-controlled and light-controlled room with free access to food and water. All experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee accepted by Faculty of Medicine; Zagazig University.

Experimental groups:

The rats were equally divided into five groups (each group includes 9 rats):

Group (I): (control group): albino rats were equally subdivided into three subgroups:

- Subgroup IA: subcutaneously injected with saline 0.5 ml/kg twice weekly for 4 weeks then the rats were sacrificed.
- Subgroup IB: orally treated with silymarin in a dose of 200 mg/kg/day for 4 weeks.
- Subgroup IC: tube fed with nanogold water (1ml/100 gm/ BW) every day for 4 weeks.
Group (II): (CCl4- treated group): injected subcutaneously with CCl4 0.5 ml/kg twice a week for 4 weeks and were sacrificed to confirm occurrence of liver fibrosis by histological examination (15).

Group (III): (CCl4-recovery group): injected subcutaneously with CCl4 0.5 ml/kg twice a week for 4 weeks then left without any treatment for another 4 weeks (16).

Group (IV): (silymarin- treated group): injected subcutaneously with CCl4 0.5 ml/kg twice weekly for 4 weeks then orally treated with silymarin in a dose of 200 mg/kg/day for 4 weeks (17).

Group (V): (Nano gold- treated group): Rats were injected subcutaneously with CCl4 0.5 ml/kg twice a week for 4 weeks and then were tube fed with 5 ppm; 1ml/100 g BW of nanogold water every day for 4 weeks (18).

Chemicals:
- Carbon tetrachloride was obtained in a liquid form from El-Gomhoria Company for Chemical and Medical Trading, Zagazig, Egypt.
- Silymarin (Legalon) was obtained in the form of capsules; each capsule contains 140 mg of silymarin produced by Chemical Industries Development (CID) GIZA-A.R.E-G.C.R.
- Nano gold was obtained from Sigma-Aldrich, Germany. The nanogold particles were manufactured by Gold NanoTech, Inc., Taipei, Taiwan, ROC using the innovative technology of physical metal miniaturization to guarantee that the nanogold was 100% gold.

At the end of the experiment, all animals were anaesthetized by phenobarbitone 35 mg/kg by intraperitoneal injection. Blood samples were collected from the orbital vein for measurement of serum ALT and AST. All livers were dissected and the specimens were immediately immersed in 10% formol saline for 24 hours to be processed for light microscopy. Paraffin sections of 5µm thickness were prepared and stained with hematoxylin and eosin (H&E) stain to study histological structure, Mallory trichrome (MT) stain to demonstrate the collagen fibers (19) and immunohistochemical stain for detection of alpha-smooth muscle actin (α-SMA) (20).

Immunohistochemical reaction was carried using avidin biotin peroxidase system. The primary antibody (α-SMA antibody) used was a mouse monoclonal antibody of IgG immunoglobulin type (Dako Life Trade -Egypt, clone 1A4, code No. M0851). It was obtained from Sigma Biochemical (St. Louis, Missouri, USA). The antigen was finally localized by the addition of DAB Chromogen. Slides were counterstained with Mayer's haematoxylin (20).

Specimens (1mm³) for electron microscope were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffered at( 4° C )for 24 hours, post-fixed in 1% osmium tetroxide for 2 hours. Specimens were embedded in epoxy resin capsules and polymerized at 60 C° for 24 hours (21). Ultrathin section were obtained using Leica Ultracut and stained with uranyl acetate and lead citrate (22, 23) then examined and photographed by JOEL EM 1010 transmission electron microscope.

Quantitative Morphometric Study:
Area % of brown colored areas of positive immunoreaction in sections stained for α-SMA was morphometrically analyzed using Leica Qwin 500 Image Analyzer Computer System (England). Also, area % of the blue colored areas of collagen fibers in MT-stained sections was analyzed.

Results were expressed as means ±SD. The data obtained by image analyzer were analyzed statistically using one-way analysis of variance (ANOVA) for
comparison between groups. ANOVA was statistically significant when P value <0.05, was considered statistically highly significant when P value <0.001 and non significant when P value >0.05 (24).

Results:

Light Microscopic Results:

Examination of H&E stained sections of all subgroups of control rats showed the same histological picture. Each hepatic lobule was formed of tightly packed cords of hepatocytes radiating from the central vein. The hepatic cords were composed of polygonal hepatocytes with rounded vesicular nuclei and acidophilic cytoplasm. Blood sinusoids with their endothelial lining were noticed between hepatocyte cords (Fig. 1). CCl4- treated group showed hepatocellular changes, hepatocytes appeared with darkly-stained nuclei and vacuolated cytoplasm. Congested dilated central veins and blood sinusoids were also observed. Some fat cells were seen between hepatocytes (Fig. 2). Meanwhile, hepatic lobules in the CCl4-recovery group were markedly affected that most hepatocytes showed darkly stained nuclei and vacuolated cytoplasm (Fig. 3).

Silymarin- treated group showed variable degrees of improvement of the changes observed in CCl4- treated group. Some hepatocytes showed vacuolated cytoplasm and darkly stained nuclei. While, other lobules revealed normal lobular architecture, as hepatocytes had vesicular nuclei and acidophilic cytoplasm radiating from central vein. Some cells were binucleated. Slightly dilated blood sinusoids were still present (Fig. 4). Nanogold- treated rats revealed relative restoring of general hepatic lobular architecture; hepatocytes were arranged as radial plates emerging from central veins. Other hepatocytes appeared with their rounded vesicular nuclei and acidophilic cytoplasm. Some cells were vacuolated and slightly dilated blood sinusoids were noticed (Fig. 5).

The portal area in the control group containing bile duct and blood vessels was seen (Fig. 6). CCl4- treated group showed portal area with cellular infiltration, bile duct proliferation and dilated congested artery (Fig. 7). CCl4-recovery group showed portal area with bile duct proliferation and dilated congested portal vein (Fig. 8). Slightly dilated components of portal areas were still present in silymarin- treated group (Fig. 9). Nanogold- treated group revealed some proliferation of bile duct in the portal area (Fig. 10).

MT- stained sections of the control group revealed few collagen fibers in the portal area (Fig. 11). Many collagen fibers were observed in the portal areas of CCl4- treated and CCl4-recovery groups (Figs. 12&13). In silymarin- treated group, few collagen fibers were demonstrated around the portal area (Fig. 14). Nanogold- treated rats revealed few collagen fibers around the central vein and portal area (Fig. 15).

Immunohistochemical stained sections of the control group showed weak positive immunoreaction for α-SMA in the wall of the central vein and in-between the hepatocytes (Fig. 16). CCl4- treated and CCl4-recovery groups showed strong positive reaction around central vein and portal area (Figs. 17&18). Mild positive immune reaction for α-SMA was seen around central vein and in- between hepatocytes in silymarin and nanogold- treated rats (Figs. 19& 20).

Electron microscope examination:

Ultrathin sections of the control group showed hepatocytes with euchromatic nuclei and prominent nucleoli. The cytoplasm contained many mitochondria, rough endoplasmic reticulum and lysosomes (Fig. 21). Hepatocytes of the CCl4- treated group clarified the light microscopic observations. They had nuclei with condensed peripheral heterochromatin, cytoplasmic vacuoles, lipid droplets and an apparent reduction of
mitochondria and rough endoplasmic reticulum. Also, CCl4-recovery group showed hepatocytes with irregular nuclei, numerous swollen mitochondria and areas of cytoplasmic loss (Figs. 22&23). Silymarin- treated group revealed the presence of apparently normal hepatocytes. Their nuclei appeared with slightly condensed peripheral heterochromatin. The cytoplasm contained mitochondria, rough endoplasmic reticulum and some vacuoles (Fig. 24). Nanogold- treated rats revealed hepatocytes nearly resumed their normal ultra structure. The hepatocytes showed euchromatic nuclei and their cytoplasm contained mitochondria and rough endoplasmic reticulum (Fig. 25).

**Figures 1 to 5 are H & E stained sections (x400):** Fig. 1: Showing tightly packed cords of hepatocytes (arrows) radiating from central vein (CV). The hepatocytes have rounded vesicular nuclei and acidophilic cytoplasm. Blood sinusoids (S) and their lining endothelium (arrows heads) are seen. (Control group) Fig. 2: Showing loss of architecture. Dilated central vein (CV) with detachment of the endothelial lining is seen. Many cells have vacuolated cytoplasm (arrow heads) and some cells have darkly-stained nuclei (curved arrow). Dilated blood sinusoids (S) and many lipid droplets (arrow) are also seen. (CCl4- treated group) Fig. 3: Showing most hepatocytes with shrunken darkly-stained nuclei (arrow) and vacuolated cytoplasm (arrow heads). Dilated central vein (CV) and dilated congested blood sinusoids (S) is observed. (CCl4- recovery group) Fig. 4: Showing slightly dilated central vein (CV). Many hepatocytes appear with vesicular nuclei and acidophilic cytoplasm (arrow) but some still have vacuolated cytoplasm and darkly-stained nuclei (arrow heads). Slightly dilated blood sinusoids (S) are still present (Silymarin- treated group) Fig. 5: Showing normal appearance of most of the hepatocytes with vesicular nuclei and acidophilic cytoplasm (arrow) radiating from central vein (CV). Slightly dilated blood sinusoids (S) are also seen. (Nanogold- treated group)

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Figures 6 to 10 are H & E stained sections (x400):

**Fig. 6:** Showing portal area containing portal vein (PV) and bile duct (d). (Control group x 200).

**Fig. 7:** Showing portal area with cellular infiltration (C), bile duct proliferation (d) and dilated congested artery (A). Hepatocytes with vacuolated cytoplasm (arrow heads) are also seen. (CCl4-treated group x 400)

**Fig. 8:** Showing portal area with bile duct proliferation (d) and dilated congested portal vein (PV). Hepatocytes with darkly stained nuclei (arrows) are also seen. (CCl4-recovery group x 400)

**Fig. 9:** Showing slightly dilated both components of portal area (P) and blood sinusoids (S). Hepatocytes with vesicular nuclei and acidophilic cytoplasm (arrow) are seen. Some hepatocytes are binucleated (arrow heads). (Silymarin-treated group x 200)

**Fig. 10:** Showing slightly dilated blood sinusoids (S) and some proliferation of bile duct (d) are also seen (Nanogold-treated group x 400)
Figures 11 to 15 are MT-stained sections (x200): Fig. 11: Showing few collagen fibers (arrow) in the portal area (P). (Control group) Fig. 12: Showing abundant collagen fibers (arrow) in portal area (P). (CCl4- treated group) Fig. 13: Showing abundant collagen fibers (arrow) in portal area (P). (CCl4- recovery group) Fig. 14: Showing few collagen fibers (arrow) around the portal area (P). (Silymarin- treated group) Fig. 15: Showing few collagen fibers (arrow) around the central vein (CV) and portal area (P). (Nanogold- treated group)
Figures 16 to 20 are sections revealing α-smooth muscle actin immunoreactivity (x400): Fig. 16: Showing weak positive immunoreaction for α-SMA in the wall of the central vein (thick arrow) and in-between the hepatocytes (thin arrow). (Control group) Fig. 17: Showing strong positive immune reaction for α -SMA around central vein (arrow). (CCl4- treated group) Fig. 18: Showing strong positive immune reaction for α -SMA (arrow) around portal area (P). (CCl4- recovery group) Fig. 19: Showing mild positive immune reaction around central vein (thick arrow) and in between cells (thin arrow). (Silymarin- treated group) Fig. 20: Showing mild positive immune reaction in-between hepatocytes (thin arrow) and around central vein (thick arrow). (Nanogold-treated group)
Figures 21 to 25 are ultrathin sections: Fig. (21): Showing a hepatocyte with euchromatic nucleus (N) and prominent nucleolus (n). The cytoplasm contains many mitochondria (m), rough endoplasmic reticulum (rr) and lysosomes (L). (Control group x Mic. Mag. 1200) Fig. (22): Showing a hepatocyte nucleus with slightly condensed peripheral heterochromatin (N). The cytoplasm contains RER (rr), electron-lucent areas (V) and some lipid droplets (L). (CCl4- treated group x Mic. Mag. 1500) Fig. 23: Showing irregular hepatocyte nucleus with condensation of peripheral heterochromatin (N). The cytoplasm contains multiple swollen mitochondria (m) and areas of cytoplasmic loss (V) are also seen. (CCl4- recovery group x Mic. Mag. 2000) Fig. 24: Showing apparently normal hepatocyte nucleus with slightly condensed peripheral heterochromatin (N). The cytoplasm contains some vacuoles (V). Mitochondria (m) and rough endoplasmic reticulum (rr) are also seen. (Silymarin-treated group x Mic. Mag. 2000) Fig. 25: Showing a hepatocyte with euchromatic nucleus (N). The cytoplasm contains mitochondria (m) and rough endoplasmic reticulum (rr) (Nanogold-treated group x Mic. Mag. 2000).

Morphometry and statistical results
The area % of both collagen fibers (MT) and α-SMA immunreaction (Immune) among the different studied groups:

The mean values of area % of collagen fibers in the liver were 0.9±0.2 in control group, 2.7±0.5 in CCl4-treated group, 11.2±2.8 in CCl4-recovery group, 1.1±0.2 in silymarin-treated group and 0.5±2.1 in the nanogold-treated group.

The mean values of area % of immunoreaction in the liver were 1±0.2 in control group, 2.9±0.5 in CCl4-treated group, 6.4±0.4 in CCl4-recovery group, 2.5±0.6 in silymarin-treated group and 2.1±0.5 in the nano group.

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Statistical analysis of the morphometrical results of the area% of both collagen fibers and α-SMA among different studied groups showed a high significant increase (P<0.001) in both CCl4-treated and CCl4-recovery groups as compared to the other groups. A significant difference was noticed between the control and silymarin in area% of α-SMA but non-significant difference was noticed between the control and nanogold-treated groups. Also, there was non-significant difference in the area% of collagen fibers between the control and silymarin or nanogold-treated groups (Table 1&Histogram 1).

Table (1): Comparison between different studied groups regarding MT and immune reaction.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>CCl4-treated</th>
<th>CCl4-recovery</th>
<th>Silymarin</th>
<th>Nanogold</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT</td>
<td></td>
<td>0.9±0.2</td>
<td>2.7±0.5</td>
<td>11.2±2.8</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>P.value</td>
<td></td>
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<td>0.003*</td>
<td>0.498</td>
<td>0.152</td>
</tr>
<tr>
<td>Immune</td>
<td></td>
<td>1±0.2</td>
<td>2.9±0.5</td>
<td>6.4±0.4</td>
<td>2.5±0.6</td>
</tr>
<tr>
<td>P.value</td>
<td></td>
<td>&lt;0.001 **</td>
<td>&lt;0.001 **</td>
<td>0.017 *</td>
<td>0.06</td>
</tr>
</tbody>
</table>

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Histogram (1): Comparison between different studied groups regarding MT and immune reaction.

Biochemical and Statistical results
Serum ALT and AST among the different studied groups:
The mean values of serum ALT were 30±7.1 in control group, 54±7 in CCl4-treated group, 75±7 in CCl4-recovery group, 45±7.1 in silymarin-group and 35±7 in the nanogold-group.

The mean values of serum AST were 35±7.1 in control group, 157±7 in CCl4-treated group, 200±7 in CCl4-recovery group, 55±7.1 in silymarin-group and 52±7 in the nanogold-group.

The results of CCl4-treated group showed a high significant level in serum activities of ALT and AST (P<0.001) in both CCl4-treated and CCl4-recovery groups as compared to the control group. A significant increase in serum activities of ALT and AST was seen in silymarin-treated as compared to the control group but non significant difference was observed in the nanogold-group (Table 2 & Histogram 2).

Table (2): Comparison between different studied groups regarding ALT and AST.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>CCl4-treated</th>
<th>CCl4-recovery</th>
<th>Silymarin</th>
<th>Nanogold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means±SD</td>
<td>Means±SD</td>
<td>Means±SD</td>
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</tr>
<tr>
<td>ALT</td>
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<td>75±7</td>
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<td>35±7</td>
</tr>
<tr>
<td>P.value</td>
<td></td>
<td>&lt;0.001 **</td>
<td>&lt;0.001 **</td>
<td>0.004*</td>
<td>0.28</td>
</tr>
<tr>
<td>AST</td>
<td>35±7.1</td>
<td>157±7</td>
<td>200±7</td>
<td>55±7.1</td>
<td>52±7</td>
</tr>
<tr>
<td>P.value</td>
<td></td>
<td>&lt;0.001 **</td>
<td>&lt;0.001 **</td>
<td>0.004*</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Histogram (2): Comparison between different studied groups regarding mean ALT and AST.

Discussion

Chronic liver diseases are considered a global challenge. Limited efficacy has been achieved by the current medical treatments for these diseases. There is a vital demand to find new strategies for the treatment of liver diseases and control of fibrogenesis process as all chronic liver diseases can lead to liver failure (25). The only curative treatment for end stage liver disease is liver transplantation. But its application is restrained by limitation of the donor organ availability and the recipient conditions (26). CCl4-induced fibrosis and cirrhosis is one of oldest and most widely used toxin based experimental model for the induction of fibrosis. It has the advantages that it mirrors the pattern of disease seen in human fibrosis and cirrhosis associated with toxic damage (27). This study was aimed to compare between the therapeutic potential of gold nanoparticles as a new line of therapy and the effect of silymarin in repairing the histological alterations in CCl4-induced liver fibrosis of adult male albino rats. Activated hepatic stellate cells (HSCs) were illustrated using α-smooth muscle actin (α-SMA) immunohistochemical stain. This reaction is a reliable and widely used as a marker to demonstrate HSC activation (28, 29).

Rats treated with CCl4 showed fatty infiltration between hepatocytes. Portal area revealed bile duct proliferation, cellular infiltration and congested portal vessels. These results were in agreement with (15, 30). Some hepatocytes contained vacuolated cytoplasm and deeply-stained nuclei. These degenerative changes denoted apoptosis according to (31). Similar degenerative changes of (CCl4) in the liver of different rodents were found. These changes varied from centrilobular necrosis and steatosis, ballooning degeneration to complete cell necrosis and infiltration of mononuclear cells in hepatic lobules. Fibrosis, cirrhosis and HCC are terminal findings (32, 33, 34). Vacuolated cells were suggested to be macrophages containing many lipid droplets (foam cells). The increased levels of ROS cause oxidative modification of low-density lipoproteins (LDLs) and formation of oxidized form which are internalized by the macrophages and implicated in the formation of foam cells. Cholesterol influx into macrophages may result in its
accumulation which promotes accumulation of lipid droplets in the cytoplasm of these foam cells. The accumulated free cholesterol induces apoptosis and finally the foam cells die \(^{(35)}\).

This group also showed dilated and congested central vein and dilated blood sinusoids with separation of the endothelial lining of the central vein. These findings were attributed to portal hypertension or to the direct effect of CCl4 on the vascular endothelial cells leading to release of endothelium relaxation factor–nitric oxide \(^{(36)}\). Cellular infiltration was also demonstrated. This inflammatory reaction could be related to oxidative stress that resulted in generation of mediators such as IL-8 and cytokine-induced neutrophil chemoattractant which attract the inflammatory cells into microcirculation and then to the liver interstitium due to destruction of the endothelial cells by the free radicals \(^{(37, 38)}\). The kinetics of fibrosis development can be divided into three phases: (1) acute injury, (2) initiation of fiber formation and (3) advanced fibrosis. The phase of acute CCl4-mediated liver fibrosis is characterized by activation of Kupffer cells and induction of an inflammatory response, resulting in secretion of cytokines, chemokines and other proinflammatory factors. This in turn attracts and activates monocytes, neutrophils and lymphocytes, which further contributes to liver necrosis \(^{(39, 40)}\).

Bile duct proliferation was evident on light microscope examination. It is reported that a ductular reaction is the proliferative response to many types of liver injuries in humans, which is seen as duct-like structures. It is generally accepted that the liver contains hepatic stem cells (progenitor cells) which was considered as a subpopulation of liver cells termed oval cells. Under the condition of severe and chronic liver injury caused by drugs, viruses, and toxins, they proliferate and differentiate both into mature hepatocytes and into biliary epithelial cells \(^{(41)}\).

MT- stained sections of CCl4-treated group revealed marked increase in collagen fibers in the portal area. This was also confirmed by morphometrical and statistical analysis. This finding was in accordance with some researchers who stated that ROS are involved in the development of hepatic fibrosis as they stimulate hepatic stellate cells (Ito cells) to proliferate and synthesize collagen \(^{(30)}\).

Transformation of HSCs from quiescent to activated phenotype precedes induction of fibrosis by 2 weeks \(^{(42)}\). The number of HSCs was found to be increased in alcoholic liver disease and in other animal models of chronic liver disease. The activated HSCs produce excess amounts of ECM components, such as laminin and collagen type IV in an accelerated fashion, resulting in fibrotic change of the liver \(^{(43)}\). The expression of \(\alpha\)-SMA was determined as a marker for the activity of HSCs, which were identified as the primary cell type that mediate fibrogenesis \(^{(44)}\). Increased collagen level was coinciding with immunohistochemical results which showed strong positive \(\alpha\)-SMA immunoreaction in the wall of central vein. These results were confirmed by morphometrical and statistical analysis which revealed that a significant increase in the area percentage of \(\alpha\)-SMA positive reaction in CCl4-treated group as compared to that of control group. This increase in the area percentage of \(\alpha\)-SMA was a reflection of increased activity of HSCs.

Electron microscope examination of the CCl4-treated group confirmed the results observed by light microscope. Hepatocytes showed few mitochondria, few rER, many lipid droplets and many irregular electron-lucent areas. These findings were in agreement with the reports of previous authors \(^{(16)}\). This structural damage was attributed to edema of the organelles. ALT and AST are important serum markers for inflammation and necrosis of the liver \(^{(45)}\). This could explain the significant increase in serum AST and ALT which were estimated in the present work, as compared to the control.
Our findings revealed that the liver structure of CCl4- recovery group was still affected. Hepatocytes had irregular nuclei and vacuolated cytoplasm. Swollen mitochondria with distorted cristae were also seen. Moreover, there was a progressive increase in the liver fibrosis confirmed by the statistical results. Similar results were obtained by previous authors. The mitochondrial swelling was related to disturbed calcium homeostasis secondary to changes in the Ca- ATPase activity and Ca content of the mitochondria. Also, the possible inhibition of Na/ K ATPase (the plasma membrane energy dependant pump) could induce mitochondrial swelling secondary to intracellular sodium accumulation and osmotic gain of water. When the level of free radicals exceeds a certain level, mitochondria try to decrease intracellular (ROS) levels by decreasing the consumption of oxygen via megamitochondria formation. Cytoplasmic vacuolation could be caused by mitochondrial degeneration which probably results from electrolyte imbalance or edema because of the inhibition of the membrane enzyme of the sodium pump. Increased lipid peroxidation of mitochondria results in inhibition of respiratory enzymes, which in turn would inhibit ATP production. The decrease in intracellular ATP leads to mitochondrial damage.

Silymarin- treated group revealed nearly normal architecture when compared with CCl4-treated group. Liver contained some vacuolated hepatocytes and few collagen fibers. The area % of collagen fibers showed a non significant statistical difference between the control group and silymarin- treated group. Hepatocytes contained normal nuclei. Concerning α-SMA immunoreaction, mild positive reaction was detected. The preserved normal structures in this group were attributed to the ability of silymarin to scavenge the free radicals before causing damage to nuclear DNA. Silymarin acts in four different ways: (1) Antioxidant as it reduce oxidative damage and was proved to be a potent-free radical scavenger and regulator of the intracellular content of glutathione (2) Cell membrane stabilizer and permeability regulator that prevents hepatotoxic agents from entering hepatocytes; (3) Promoter of ribosomal RNA synthesis, stimulating liver regeneration and (4) Inhibitor of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibers leading to cirrhosis. (5) Other mechanisms: anticarcinogenic properties have also been documented. Silymarin was found to reduce the proliferation of stellate cells isolated from fresh liver of rats by about 75% and down regulates gene expression of extracellular matrix components in dispensable for fibrosis. Silymarin can efficiently reduce intracellular ROS levels of hepatocytes, thus preventing oxidative stress-induced cellular damage. Furthermore, hepatic cell proliferation was found to be stimulated after silymarin treatment suggesting that enhanced liver regeneration may help replace the damaged liver cells. For at least the past 2 centuries, silymarin remains highly regarded as a safe and effective hepatoprotective medication. Beside its antioxidant properties silymarin has antifibrotic, immunomodulating and anti-inflammatory effects. Furthermore, it was found that silymarin nanoparticles had a protective effect against liver glutathione depletion and lipid peroxidation.

Examination of the liver tissue of rats treated with gold nanoparticles revealed a preserved architecture when compared with CCl4-treated group. Concerning α-SMA immunoreaction, mild positive reaction was detected. This reaction showed a non significant statistical difference between the control group and nanogold- treated group. Following systemic administration, conventional NPs are opsonized by plasma proteins, known as foreign bodies and rapidly captured by the reticuloendothelial system (RES). The major organs of accumulation of NPs are the liver and the spleen.
due to their rich blood supply and numerous tissue-resident phagocytic cells, consequently liver targeting by NPs may be favorable for treating liver diseases \(^{(60)}\). It is reported that hydrodynamic size of nanoparticles (NPs) also affects NPs clearance from circulation \(^{(61)}\). Different HSC-selective nanoparticles (NP) carriers can reduce liver fibrosis based on the conjugation of targeting ligands directed against several receptors expressed by activated HSCs at the surface \(^{(14)}\). Nanoparticles act through down regulation of hepatic stellate cells and attenuation of Kupffer cells \(^{(62)}\). Studies using NanoCurc™ to treat animals with hepatic injury and fibrosis induced by CCl4 administration showed attenuated hepatocellular injury and levels of pro-inflammatory cytokines, prevented hepatic fibrosis and induced HSC apoptosis. NanoCurc™ inhibits pro-fibrogenic transcripts associated with activated myofibroblasts \(^{(63)}\). Another mechanism by which nanoparticles act is peroxisomal proliferator activated receptor (PPARs), which belong to the super- family of nuclear hormone receptors with transcriptional activity controlling multiple processes, have been implicated in liver fibrogenesis \(^{(64, 65)}\). The fullerene nanoparticles were also known to selectively enter cells damaged due to oxidative stress and potentially inhibited apoptosis by hindering the stress activated protein kinases \(^{(66)}\).

The ability of gold nanoparticles in inhibiting lipid peroxidation thereby preventing the ROS generation has restored the imbalances in the antioxidants and liver enzymes responsible for the cell dysfunction and destruction \(^{(67, 68)}\). However, toxicity associated with nanomaterials should be considered before NPs are widely utilized as drug delivery systems, especially for inorganic nanoparticles \(^{(69)}\). Higher NP concentrations (200 nM) reduced cell viability mostly through induction of reactive oxygen species, which was significantly induced at concentrations of 50 nM or higher. At these concentrations, both actin and tubulin cytoskeleton were deformed and resulted in reduced cell proliferation and cellular differentiation. At 10 nM, no significant effects on any cellular parameter could be observed. These data highlight the importance of using multiple assays to cover the broad spectrum of cell–NP interactions and to determine safe NP concentrations \(^{(70)}\).

**Conclusion**

Both silymarin and gold nanoparticles were effective in ameliorating CCl4-induced liver fibrosis and parenchymal changes in adult male albino rats. However, gold nanoparticles proved to be more effective.

**Recommendation**

In advanced cases of fibrosis which are not responding to medical treatment, gold particles could be considered as they proved to be experimentally effective. However, their administration to human patients needs large-scale controlled and double-blinded clinical trials with different sizes, doses and durations to avoid any hazardous risks. Experimental studies using combined silymarin and gold particles are also recommended.

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Authors’ contributions

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