Original Article

# Histological and Immunohistochemical Studies on the Effect of Curcumin Nanoparticles Versus Mesenchymal stem cells on Acetaminophen-Induced Acute Liver injury in Adult Male Albino Rats

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## ABSTRACT

**Introduction:** Acute liver injury (ALI) induced by acetaminophen, a prevalent trouble, may cause acute liver failure. Curcumin possesses antioxidant, anti-inflammatory and antifibrotic properties. While its poor solubility and rapid metabolism cause a limited therapeutic effect. Nano-formulation might overcome this shortage and enable enhanced properties. Mesenchymal stem cells (MSCs) therapy has received great attention through offering multiple pleiotropic effects for tissue repair and regeneration in diversity of research. This could be mediated through their inhibition of oxidative-stress, inflammation, and fibrosis as well as their paracrine trophic effect.

**Aim of the Work:** Comparing the probable therapeutic impact of curcumin nanoparticles versus MSCs injection in acetaminophen ALI rat model.

**Materials and Methods:** Thirty-four male albino rats aged 3 months were splited into donor group, group I (Control), group II (Acetaminophen), group III (Recovery), group IV (Acetaminophen + Nanocurcumin) & group V (Acetaminophen + MSCs). At the end of experimental duration, biochemical analysis [for serum alanine transferase (ALT) albumin, total bilirubin, and tissue malondialdehyde (MDA)], histological, immunohistochemical studies [for glial fibrillary acidic protein (GFAP) & alfa-smooth muscle actin (α-SMA)], and statistical assessments were performed.

**Results:** Acetaminophen group recorded high ALT, bilirubin & liver MDA levels and low albumin values. While there was insignificant increase in collagen fiber deposition, GFAP & α-SMA immunoreactivity versus the control. In addition, disorganized hepatic lobule architecture, some hepatocytes with darkly stained nuclei & cytoplasmic vacuolation, and inflammatory infiltration were observed. The recovery group illustrated marked aggravation of previously stated findings. However, treatment with curcumin nanoparticles and MSCs ameliorated hepatocytes injury, oxidative stress, inflammation, and fibrosis with MSCs superiority.

**Conclusion:** Both curcumin nanoparticles and MSCs treatment exerted a therapeutic effect against acetaminophen hepatotoxicity. However, MSCs treatment provoked better influences on restoring hepatocellular structure and function.

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**Key Words:** α-SMA, acute liver injury, curcumin nanoparticles, GFAP, MSCs.

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## INTRODUCTION

Worldwide, drug-induced hepatic injury is a serious clinical issue. Acetaminophen recognized as paracetamol or N-acetyl-p-aminophenol (APAP) mostly induces acute injury of liver tissue either due to accidental or intentional overdose[1]. It is a common disorder that industriously threatens patients' health and life as it could proceed into liver failure, and death in most countries[2]. The main pathological finding is a sharp drop in hepatic function because of necrosis of multiple hepatocytes[3].

Acetaminophen has analgesic, anti-inflammatory and antipyretic effects[4]. It is greatly available and utilized by all ages[5]. During its metabolic process, the cytochrome P450 liver enzyme transforms acetaminophen into a reactive product, N-acetyl para-benzoquinone imine (NAPQI), that combines with cells proteins resulting in liver tissue injury[6].

Recently, the natural products application for management of liver injury is demonstrated as a promising approach[7]. Curcumin is a hydrophobic component in the curcuma longa plant. It shows a great concern for the diversity of its effects[8]. This includes antioxidant, antimicrobial, antifibrosis, immunomodulatory & anti-inflammatory effects[8,9].

While curcumin reduced water solubility, low bioavailability, and fast metabolism appear as main obstacles for its effective therapeutic impacts. Hence, investigators have attempted to explore new effective delivery methods[10]. Nanocurcumin has been developed to augment the therapeutic effects of curcumin. It was proved

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that the pharmaceutical formulations of nanocurcumin keep its pharmaceutical efficacy and enhance the bioavailability than conventional curcumin[11].

The great tendency to use acetaminophen, a commonly consumed analgesic, in high doses for faster effects is of substantial concern as it increases the risk of developing acute liver failure. Such failure may be very severe, necessitating liver transplantation[5]. However, the shortage of donors, operative and postoperative complications, high costs, and immuno-suppression decrease its application[12]. Thus, an imperative need to detect appropriate therapeutic methods is essential to avoid proceeding of liver damage and promote tissue repair. Accordingly, the use of regenerative medicine modality as cell-based treatment is considered as hopeful targets in clinical conditions. Such promising technology paves as a target of potentially curative remedies for some of humanity's fatal diseases[13].

Mesenchymal stem cells (MSCs) are multipotent self-renewing & undifferentiated cells which promote repair of injured tissues. MSCs can be found in adipose tissue, bone marrow, blood, & umbilical cord[14].

The MSCs have been widely applied in different research as they have favorable properties that show hopeful results. This could be mediated by the multiple sources for isolation, easy extraction methods, simple culture, expansion, and store. In addition, they are able to self-renew, migrate to areas of tissue damage with trans-differentiation into different cells, besides production of variable factors like exosomes and growth factors and their immunomodulatory properties[15,16].

## AIM OF THE WORK

Therefore, this current work aimed at evaluation of the possible therapeutic impact of curcumin nanoparticles versus MSCs injection in experimentally induced ALI by acetaminophen in adult rats.

## MATERIALS AND METHODS

### *Materials*

#### Chemicals

Adol (Paracetamol) 500mg tablets were purchased from the Gulf Pharmaceutical Industries in Ras AI Khaimah, U. A. E. Each tablet was crushed and dissolved in 10ml isotonic saline (0.9% NaCl)[17].

#### Adipose derived mesenchymal stem cells (ADMSCs)

Labelled ADMSCs with Paul Karl Horan-26 (PKH-26) were prepared at Histology Department, Faculty of Medicine, Cairo University. PKH26-labeled ADMSCs were provided as $1 \times 10^6$ ADMSCs in 0.5 ml phosphate buffer saline (PBS) for every animal.

#### Curcumin nanoparticles

Curcumin nanoparticles, NT-Cur-NPs, [product code: 4020] were obtained from Nano-Tech Egypt of photo-electronics, 6th October City, Egypt in the form of dried yellowish brown powder in a well-sealed container.

#### Animals

Thirty-four male albino rats, (~3 months old, ~200 gm weight) were utilized. At the Animal House, Faculty of Medicine, Cairo University, rats were housed in cages, and allowed for regular chow and water. The animals were maintained for 48 hours in the prior conditions before beginning the study to allow them to adapt to their new condition. All steps were accomplished according to Cairo University-Institutional Animal Care & Use Committee guidelines (approval number: CU III F 17 22).

Animals were distributed into:

Donor group, 2 rats: They were subjected to ADMSCs isolation, culture, phenotyping and labeling.

**Group I** (Control group), 10 rats:

These rats were furtherly divided into 4 subgroups:

- Subgroup Ia (2 rats): The rats received nothing throughout the whole experimental duration (15 days).
- Subgroup Ib (4 rats): Each rat received once intraperitoneal (IP) injection of 2 ml isotonic 0.9% NaCl. Following 24 hours two rats were euthanized, the other two rats were euthanized on day 15 of the experimental study corresponding to groups II and III respectively.
- Subgroup Ic (2 rats): Each rat was treated as in subgroup Ib. After 24 hours; it received 1 ml isotonic saline solution orally by gavage once per day for 2 weeks. Sacrifice of rats was performed at day 15 of the experimental study corresponding to group IV.
- Subgroup Id (2 rats): Rats were treated as subgroup Ib. Twenty-four hours later; the rat injected once with 0.5 ml PBS via tail vein. Rats were sacrificed at day 15 of the experiment corresponding to group V.

**Group II** (Acetaminophen group), 5 rats:

Each animal was injected once with acetaminophen (500 mg/kg) dissolved in 2 ml isotonic solution through intraperitoneal route, then sacrificed after 24 hours[17] to ensure liver injury.

**Group III** (Recovery group), 5 rats:

Rats received a single dose of acetaminophen the same as group II and kept with no treatment, then euthanized at day 15 of the experiment (at the same time as Acetaminophen + Nanocurcumin group and Acetaminophen + MSCs group).

**Group IV** (Acetaminophen + Nanocurcumin group), 5 rats:

Rats were treated the same as in group II. Twenty-four hours later (day 2 of the experiment), curcumin nanoparticles at 50 mg/kg were dissolved in isotonic saline solution and given once daily for 2 weeks through oral administration by gavage[18]. Each rat received daily 10 mg of curcumin nanoparticles dissolved in 1 ml isotonic

saline solution. On day 15 of the experiment animals were euthanized.

**Group V** (Acetaminophen + MSCs group), 7 rats:

Animals were treated as in acetaminophen group. Following twenty-four hours (day 2 of the experimental study), each rat injected once with $1 \times 10^6$ PKH26-labeled ADMSCs in 0.5 ml PBS through tail vein[19].

Two rats were chosen randomly and sacrificed after 3 days (day 5 of the experiment) to confirm stem cells homing at liver[20]. While the remaining 5 animals were euthanized on day 15 of the experiment.

## *Methods*

### Preparation of ADMSCs

Collected adipose tissue (5 gm) was obtained from rat's perirenal tissue and minced in culture plate using sterile scissors. Then Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 12491015, Gibco, UK) including collagenase type 1 (CAS 9001-12-1, Sigma Aldrich, USA) was applied to the minced tissue at 37°C and for 120 minutes on a shaker. Centrifugation at 1,200 g for ten minutes was followed. Then the floating part was thrown, and the pellet was transferred to DMEM including 10% (volume/volume) fetal bovine serum & 1% (volume/volume) penicillin/ streptomycin. Then culturing of pellets until confluency was approximately 80%. MSCs at passage 2 were used in the experiment[21]. ADMSCs preparation, isolation, and centrifugation were accomplished at Department of Histology, Faculty of Medicine, Cairo University.

### Labelling of ADMSCs

For labelling with PKH26, incubation of ADMSCs were carried in cocktail formed of DMEM medium (without serum) completed with PKH26 solution (Cat. No. MIN126, Sigma, USA). This incubation was kept for one hour in a humidified incubator (37°C, 5% $CO_2$)[22].

## *Animal studies*

### Serum biochemical studies

Blood samples were collected from tail vein before sacrifice & analyzed to detect serum level of:

a. Alanine aminotransferase (ALT) to detect hepatic injury.

b. Albumin to assess secretory function of liver.

c. Total bilirubin as an indicator of liver excretory function.

These were assessed using the colorimetric method according to the standard manufacture instructions of BioAssay Systems Company (California, USA).

### Animal sacrifice

At each time point, the rats were sacrificed after anesthesia with IP injection of ketamine (80 mg/kg) / xylazine (10 mg/kg)[23]. Then the right hepatic lobes excision was performed.

### Tissue biochemical studies

Small hepatic fragments (about 2.5 cm3) were dissected for homogenization and detection of tissue malondialdehyde (MDA) levels, oxidative stress marker. This was achieved by colorimetric method using Biodiagnostic kits (Biodiagnostics, Giza, Egypt).

Blood samples and tissue homogenate biochemical analysis were carried out in Biochemistry Department, Faculty of Medicine, Cairo University.

## *Histological studies*

Small fragments from rats' right hepatic lobes (about 1cm3) were excised for paraffin blocks preparation. Serial sections of 5 µm thick were cut for:

i. Fluorescent microscopic study: to detect the homed PKH26 labeled stem cells in unstained hepatic sections of group V by fluorescent microscope Olympus BX50F4.

ii. Light microscopic study:

   a. Hematoxylin and Eosin stain (H&E): For demonstration of liver histological architecture & changes[24].

   b. Masson's trichrome stain: To illustrate deposited collagen fibers[24].

   c. Immunohistochemical staining using the following antibodies[24]:

      - Glial fibrillary acidic protein (GFAP), mouse monoclonal antibody [product number G3893, Sigma Aldrich, USA]: a specific marker for hepatic stellate cells (HSCs)[25]. It appears as cytoplasmic immunoreaction.
      - Alfa-smooth muscle actin (α-SMA), rabbit polyclonal antibody [product number ABT1487, Sigma Aldrich, USA]: a marker of activated fibrogenic cells myofibroblasts[26]. It appears as cytoplasmic immunoreactivities.

Using the avidin-biotin technique immunostaining[24], antigen retrieval was accomplished via heating up liver sections in 10 mM citrate buffer (cat no 005000) pH 6 until ten minutes passed. After that liver sections were left to cool for 20 minutes at room temperature. This was followed by incubation with the primary antibodies for one hour. According to instructions of manufacturer, the recommended dilutions were 1:400-1:800 for GFAP antibody and 1:1,000 for α-SMA antibody. Staining was done using the Ultravision One Detection System (catalogue number TL - 060- HLJ) and Lab Vision Mayer's hematoxylin counterstaining (catalogue number TA- 060-MH). Ultravision One Detection System, Citrate buffer & Ultravision Mayer's hematoxylin were obtained from Labvision, ThermoFisher scientific, USA[24].

The positive control for GFAP and α-SMA appeared as cytoplasmic immunoreaction in the brain tissue and smooth muscle respectively. However, negative control liver sections were prepared with skipping primary antibodies application.

### *Morphometric Study*

Ten non-overlapping fields (x400 magnification) from 5 sections of 5 rats in each group were examined by a light microscope connected to a colored video camera. The area percent was obtained by "Leica Qwin 500C" an image analyzer computer system (Leica Imaging System Ltd, Cambridge, UK) for detection of collagen deposition stained with Masson's trichrome stain, GFAP & α-SMA positive immunoreactions in corresponded immune-stained sections.

All histological and morphometric studies were performed at the Histology Department, Faculty of Medicine, Cairo University.

### *Statistical Analysis*

The results obtained were evaluated using Statistical Package for Social Science (SPSS) version 16 (SPSS, Chicago, USA). One-way analysis-of-variance (ANOVA) followed by post Hoc Tukey test was used to compare values among various groups. The findings were demonstrated as mean ± standard deviation. The variance was significant when the probability (*P*) value was less than 0.05 and highly significant when the *P value* was less than < 0.01[27].

## RESULTS

### *General observations*

- No mortality was recorded in the present study.
- Lowered activity was noticed in the rats of groups II and III.
- The control subgroups (subgroup Ia, Ib, Ic & Id) exhibited similar biochemical and histological results at different sacrifice points, so they were all termed as control group (Group I).

### *Biochemical results*

Serum ALT and total bilirubin and tissue MDA levels (Figures 1 a,b,c) demonstrated a highly significant rise in acetaminophen & recovery groups versus control (*P value* 0.000). In addition, they detected a highly significant boost in recovery group versus acetaminophen group (*P value* 0.000). Conversely the mean values in groups IV & V displayed a highly significant diminution versus group III (*P value* 0.000). Groups IV & V recorded a highly significant increase in serum ALT and tissue MDA levels in comparison to group I (*P value* 0.000), as well as a highly significant elevation in the total bilirubin versus the control group (*P value* 0.001). Moreover, group V recorded a highly significant decrease in ALT, total bilirubin and MDA levels versus group IV (*P value* 0.000).

Serum albumin level (Figure 1b) illustrated a highly significant decrease in group II and group III than control (*P value* 0.000). Additionally, a highly significant reduction in group III versus group II was recorded (*P value* 0.000). On the contrary, groups IV and V illustrated a highly significant rise in comparison to recovery group (*P value* 0.000). In addition, they exhibited a highly significant decrease versus control group (*P value* 0.000). Furthermore, group V recorded a highly significant rise in albumin compared to group IV (*P value* 0.001).

### *Histological Results*

**Fluorescent labelled sections**

The unstained liver sections of group V (stem cells-treated group, rats sacrificed at day 5) showed red fluorescence of MSCs labeled with PKH26 that appeared in parenchyma of hepatic tissue confirming their homing in liver (Figure 2).

**H&E-stained sections**

The control group, (Figures 3 a,b) revealed liver cords radiating from central vein & separated by sinusoidal capillaries that were lined by endothelial cells & von Kupffer cells. Hepatocytes were polyhedral in shape with an acidophilic granular cytoplasm and spherical pale centrally located nuclei. Binucleation was noticed in some cells. Portal tracts were seen at corners of lobules.

In Group II [Paracetamol group] (Figures 3 c,d), hepatic tissue demonstrated disorganized hepatic lobule architecture. Some hepatocytes showed cytoplasmic vacuolation and deeply stained nuclei. There was congestion in central vein & portal tract vasculature, and dilatation of blood sinusoids. Besides, there was severe inflammatory cells infiltration near the central vein.

Sections of group III [Recovery group] (Figures 3 e,f) illustrated congested central vein and markedly disrupted hepatocytes & hepatic lobule architecture. Ballooning of multiple hepatocytes with cytoplasmic vacuolations and pyknotic nuclei were detected. Some fields showed congested blood sinusoids and congested portal tract vasculature. Additionally, few inflammatory cells were observed within the portal tract.

In group IV (Acetaminophen + Nanocurcumin group) (Figures 3 g,h), apparently less-organized hepatic plates extending from central vein and separated with blood sinusoids. Some hepatocytes had pale nuclei either single or binucleation, while others appeared with vacuolated cytoplasm and darkly stained nuclei. Non congested central vein, blood sinusoids & portal tract vasculature along with few inflammatory cells within the portal tract were detected.

Group V (Acetaminophen + MSCs) (Figures 3 i,j) exhibited nearly well-organized hepatic architecture. Most hepatocytes were apparently normal with vesicular single nuclei or binucleation radiating from non-congested central vein. However, a few hepatocytes with vacuolated

cytoplasm and a few inflammatory cells in portal tract were noted. The portal vasculature appeared non congested.

**Masson's trichrome stained sections**

Control group (Figures 4 a,b) exhibited scanty collagen deposition around central vein & in portal tract. Group II [Paracetamol group] (Figures 4 c,d) revealed few collagen fibers deposition, while in group III [Recovery group] (Figures 4 e,f) the collagen deposition was marked around the portal tract. In group IV (Acetaminophen + Nanocurcumin group) (Figures 4 g,h) there was some collagen fibers deposition. While in group V (Acetaminophen + MSCs group) (Figures 4 i,j), the collagen deposition was few around central vein & in portal tract.

**GFAP immunohistochemically stained sections**

Regarding the negative control hepatic sections (Figures 5 a,b), negative immunostaining was illustrated in liver fields of central vein and portal tract with skipping the step of adding GFAP primary antibody.

In group I (control group) (Figures 5 c,d) showed few GFAP immune-positive hepatic stellate cells (HSCs) with cytoplasmic expression and long processes around the blood sinusoids within central vein area and portal area. As regards group II [Paracetamol group] (Figures 5 e,f), some GFAP immune-positive HSCs were seen around the blood sinusoids. However, group III [Recovery group] (Figures 5 g,h) illustrated abundant GFAP immune positive HSCs around the blood sinusoids. This immune expression was some in group IV (Acetaminophen + Nanocurcumin group) (Figures 5 i,j) and few in group V (Acetaminophen + MSCs group) (Figures 5k,l).

**α-SMA immunohistochemically stained sections**

The negative control liver sections (Figures 6 a,b) after skipping the addition of α-SMA primary antibody demonstrated negative immunostaining in central vein and portal tract fields.

Hepatic sections of group I (Figures 6 c,d) illustrated very few α-SMA immune-positive cells with cytoplasmic immunostaining around central vein & within portal area. As regards group II [Paracetamol group] (Figures 6 e,f), few α-SMA immune-positive cells were noticed around central vein, blood sinusoids & within portal area. These immune-positive cells were abundant in group III [Recovery group] (Figures 6 g,h) While group IV (Acetaminophen + Nanocurcumin group) (Figures 6 i,j) showed some α-SMA immune-positive cells and group V (Acetaminophen + MSCs group) (Figures 6 k,l) demonstrated few α-SMA immune-positive cells.

## *Morphometric results*

The mean area percent of collagen deposition stained by Masson trichrome stain, GFAP & α-SMA immunostaining (Figures 4k,5m,6m) illustrated a non-significant rise in acetaminophen group versus control group. The *P value* was 0.381, 0.113, 0.108 as regards the mean area percent of collagen deposition, GFAP & α-SMA immunostaining respectively. There was a highly significant boost in recovery group versus group I and group II (*P value* 0.000). However, in groups IV and V, the mean values recorded a highly significant diminution in comparison to group III (*P value* 0.000). Moreover, there was a highly significant rise in groups IV and V versus the control group (*P value* 0.000). Comparing group V with group IV a highly significant decrease was observed, statistically the *P value* was 0.000 in analysis of collagen deposition & GFAP immunostaining and 0.003 in analysis of α-SMA immunostaining.

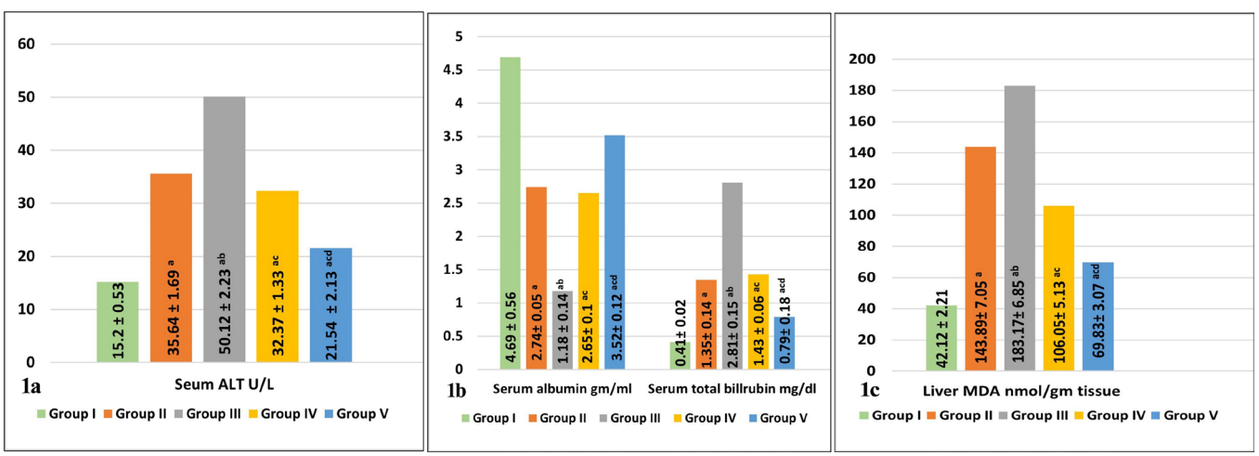


**Fig. 1:** Showing mean values of: 1a: Serum ALT level. 1b: Serum albumin and total bilirubin levels. 1c: Liver MDA level. [a as compared to group I, b as compared to group II, c as compared to group III & d as compared to group IV (highly significant difference at $P < 0.01$)]

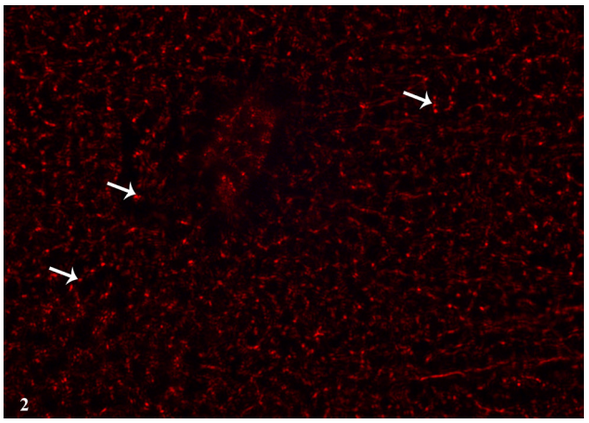


**Fig. 2:** Photomicrograph of unstained liver section from group V (Acetaminophen + MSCs group, sacrificed at day 5) showing red fluorescence of MSCs labeled by PKH26 (arrows) in liver parenchyma. [Fluorescent microscope, × 200]

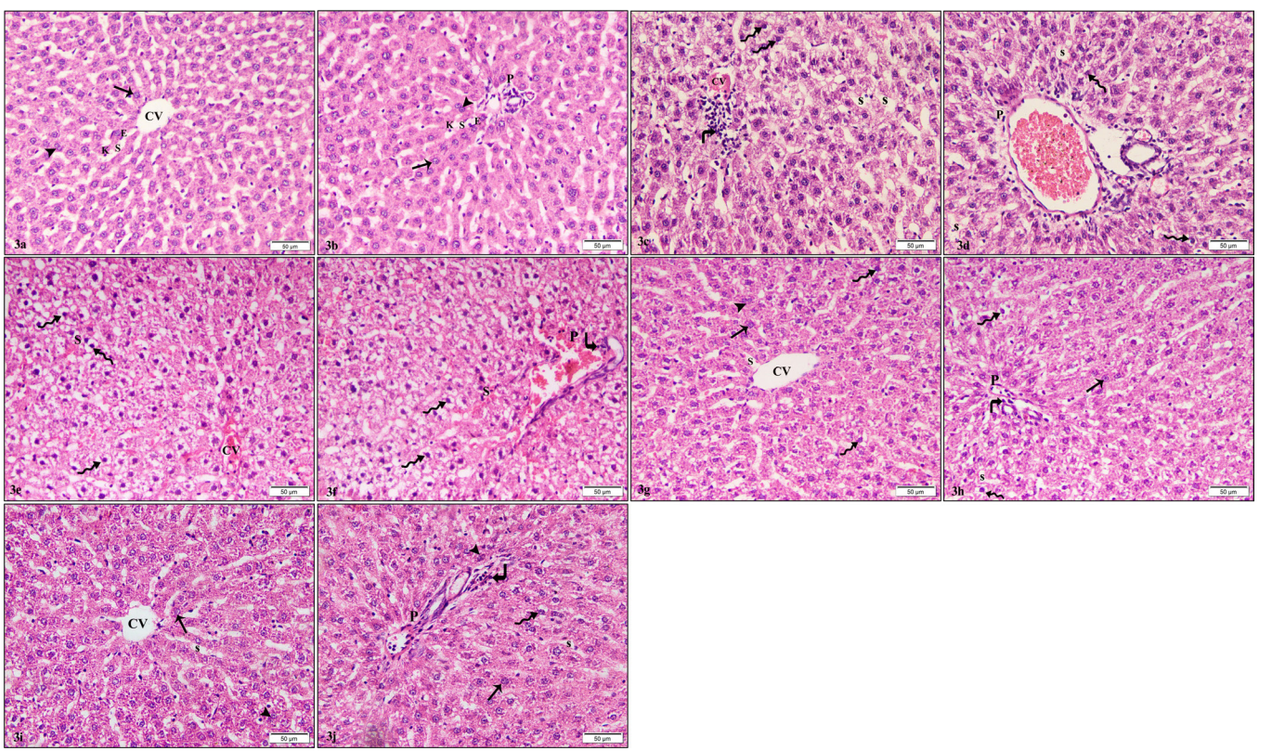


**Fig. 3:** Photomicrographs of H&E-stained liver sections of: 3a&3b group I (The control group): Illustrating hepatocytes distribution around central vein (3a) and portal tract (3b). Plates of hepatocytes are extended from central vein (CV) and separated by sinusoidal capillaries (S) lined by endothelial cells (E) & Kupffer cells (K). Hepatocytes are polyhedral in shape, showing eosinophilic granular cytoplasm with central spherical pale nuclei (straight arrow) and some are binucleated (arrowhead). A portal tract (P) is demonstrated. 3c&3d group II (Paracetamol group): Showing hepatocytes distribution around central vein (3c) and portal tract (3d). Disorganized hepatocytes and hepatic lobule architecture are seen. Some hepatocytes illustrate deeply stained nuclei (wavy arrows) (in 3c) and deeply stained nuclei and cytoplasmic vacuolations (wavy arrows) (in 3d). Congested central vein (CV), dilated blood sinusoids (S) and congested portal tract vasculature (P) are recognized. In addition, heavy inflammatory cells infiltration (right angle arrow) is noticed near the central vein (CV) (in 3c). 3e&3f group III (Recovery group): Illustrating hepatocytes distribution around central vein (3e) and portal tract (3f). Marked disorganized hepatocytes and hepatic lobule architecture are seen around congested central vein (CV). Multiple ballooned hepatocytes having cytoplasmic vacuolations & pyknotic nuclei (wavy arrows) are detected & separated by congested sinusoids (S). Portal tract vessel (P) appeared congested as well as few inflammatory cells (right angle arrow) are also observed (in 3f). 3g&3h group IV (Acetaminophen + Nanocurcumin group): Demonstrating hepatocytes distribution around central vein (3g) and portal tract (3h). Less organized hepatic cords with some apparently normal hepatocytes (straight arrow) are seen radiating from central vein (CV). Binucleated hepatocytes (arrowhead) can be detected (in 3g). Other liver cells (wavy arrows) exhibit either darkly stained nuclei and/or cytoplasmic vacuolations. Notice the non-congested central vein (CV), sinusoidal capillaries (S) and portal tract vasculature (P). Few inflammatory cells (right angle arrow) within the portal tract are observed (in 3h). 3i&3j group V (Acetaminophen + MSCs): showing hepatocytes distribution around central vein (3i) and portal tract (3j). Well organized liver architecture is seen. Apparently normal hepatocytes radiate from the central vein (CV) & are separated by blood sinusoids (S). Liver cells exhibit either single pale nuclei (straight arrow) or binucleation (arrowhead). Few hepatocytes have vacuolated cytoplasm (wavy arrow) (in 3j). In addition to the presence of non-congested portal tract vessels (P) and few scattered inflammatory cells (right angle arrow) within portal tract. [H&E, x200]

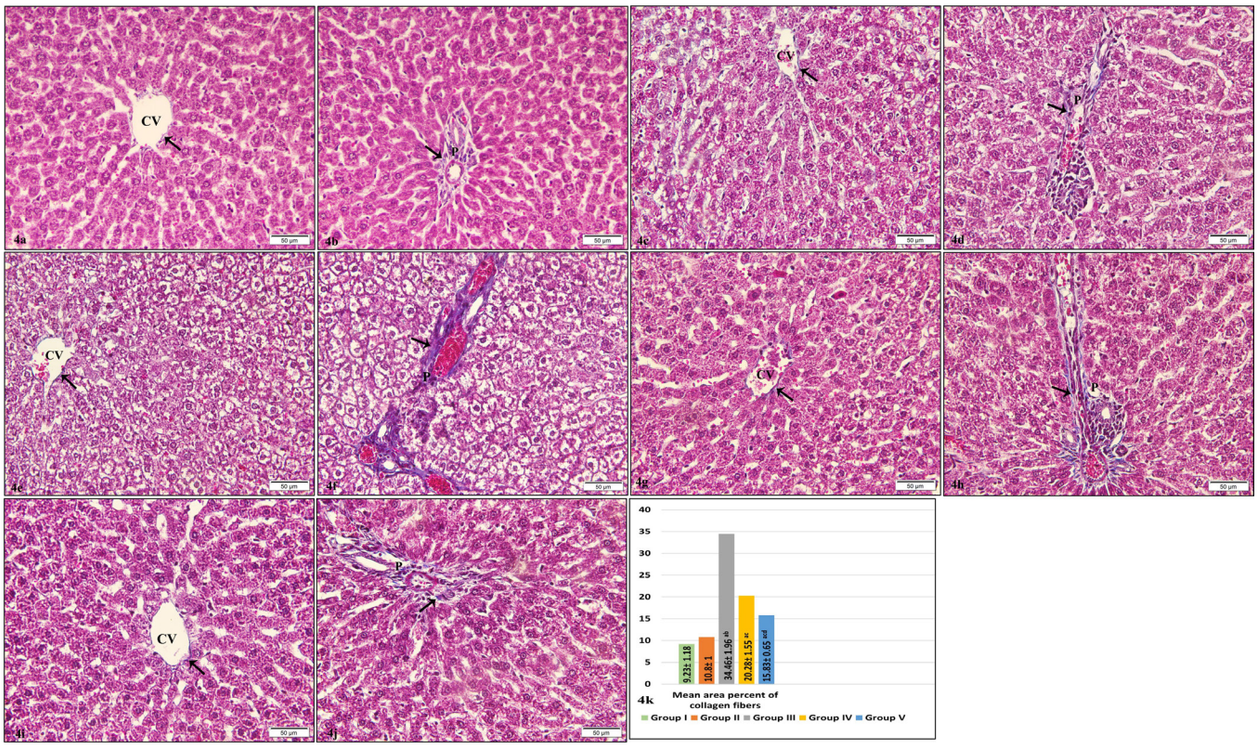


**Fig. 4:** Photomicrographs of Masson's trichrome-stained sections of liver of: 4a &4b group I (The control group): Illustrating scanty collagen fiber distribution (arrow) around central vein (CV) (in 4a) & in portal tract (P) (in 4b). 4c&4d group II (Paracetamol group): Showing a few amount of collagen deposition (arrow) around the central vein (CV) (in 4c) & in the portal tract (P) (in 4d). 4e&4f group III (Recovery group): Illustrating collagen fibers deposition (arrow) around the central vein (CV) (in 4e) and abundant collagen fibers (arrow) in portal tract (P) (in 4f). 4g&4h group IV (Acetaminophen + Nanocurcumin group): Showing some deposited collagen fiber (arrow) around the central vein (CV) (in 4g) & in portal tract (P) (in 4h). 4i&4j group V (Acetaminophen + MSCs group): Illustrating few collagen fiber deposition (arrow) around the central vein (CV) (in 4i) & in portal tract (P) (in 4j). [Masson's trichrome stain, x200]. 4k: Histogram demonstrating the mean area percent of collagen fibers stained with Masson's trichrome. [a as compared to group I, b as compared to group II, c as compared to group III & d as compared to group IV (highly significant difference at $P < 0.01$)]

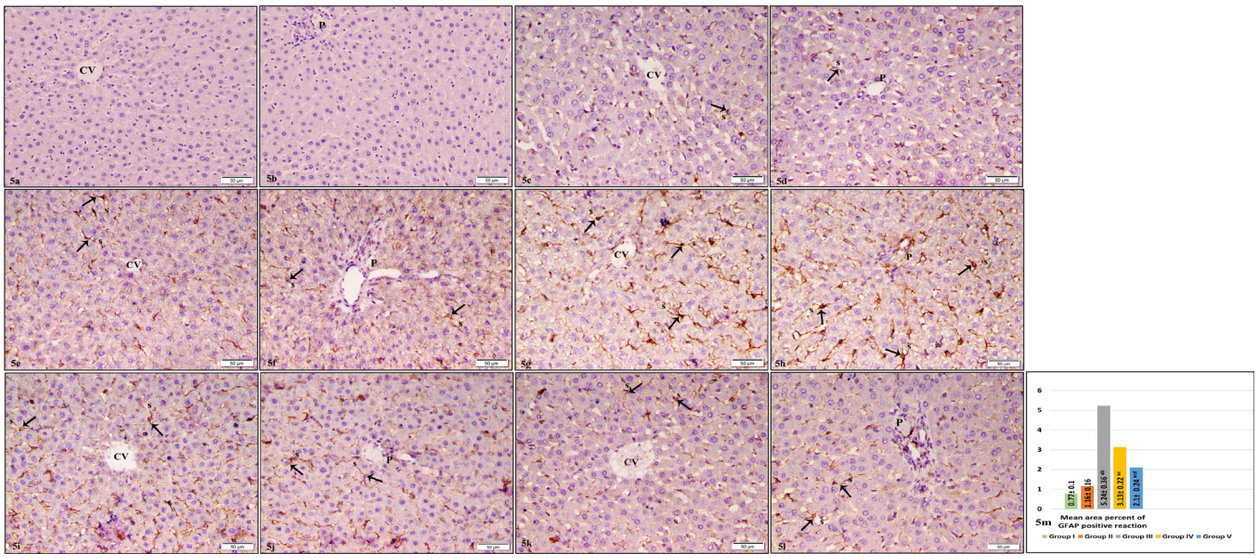


**Fig. 5:** Photomicrographs of GFAP immunohistochemically stained liver sections of: 5a&5b negative control liver sections after skipping the GFAP primary antibody illustrating negative immunostaining in central vein (CV) field (in 5a) & portal tract field (P) (in 5b). 5c&5d group I (The control group): Demonstrating a few positive GFAP immunostaining in cytoplasm and long processes of HSCs (arrow) around the blood sinusoids (S) in the central vein (CV) field (in 5c) & portal tract field (P) (in 5d). 5e&5f group II (Paracetamol group): Showing some GFAP immune-positive HSCs (arrows) around the blood sinusoids (S) in the central vein (CV) field (in 5e) & portal tract field (P) (in 5f). 5g&5h group III (Recovery group): Abundant GFAP immune-positive HSCs (arrows) are detected around the blood sinusoids (S) in the central vein (CV) field (in 5g) & portal tract field (P) (in 5h). 5i&5j group IV (Acetaminophen + Nanocurcumin group): Some GFAP immune-positive HSCs (arrows) are seen around the blood sinusoids (S), in the central vein (CV) field (in 5i) and portal tract field (P) (in 5j). 5k&5l group V (Acetaminophen + MSCs group): Few GFAP immune-positive HSCs (arrows) are observed around the blood sinusoids (S), in the central vein (CV) field (in 5k) and portal tract field (P) (in 5l). [GFAP immunostaining, x200]. 5m: Histogram illustrating the mean area percent of GFAP positive reaction. [a as compared to group I, b as compared to group II, c as compared to group III & d as compared to group IV (highly significant difference at $P < 0.01$)]

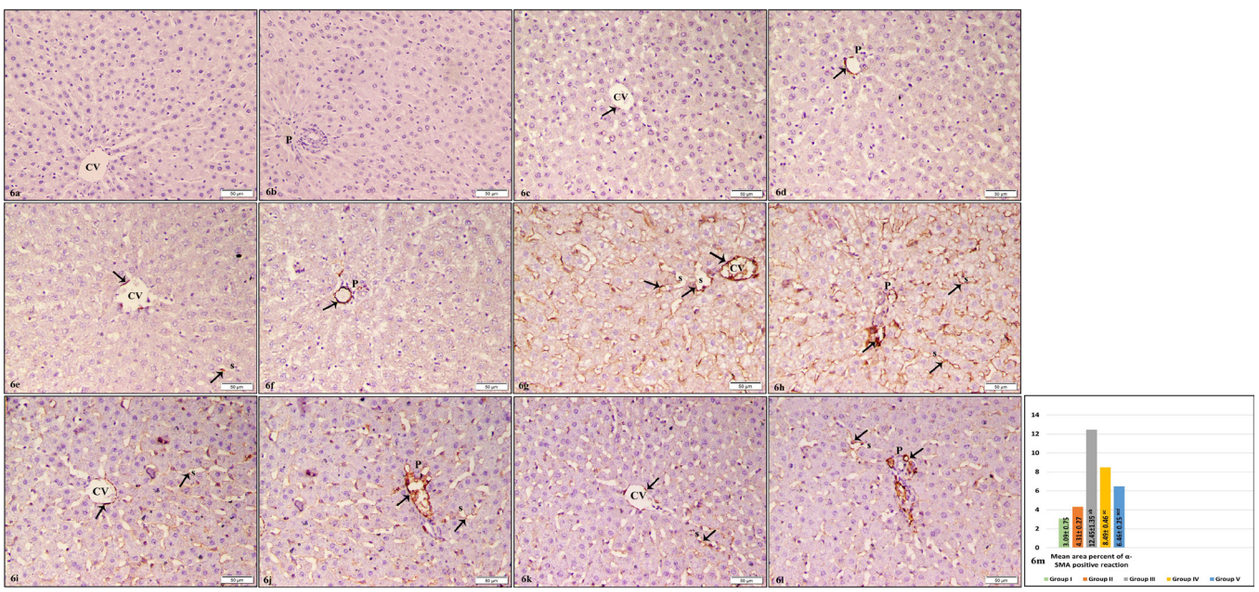


**Fig. 6:** Photomicrographs of α-SMA immunohistochemically stained liver sections of: 6a&6b negative control liver sections with skipping the α-SMA primary antibody showing negative immunostaining in the central vein (CV) field (in 6a) & portal tract field (P) (in 6b). 6c&6d group I (The control group): Cytoplasmic immunostaining is seen in very few α-SMA immune-positive cells (arrow) around central vein (CV) (in 6c) and within portal area (P) (in 6d). 6e&6f group II (Paracetamol group): Few α-SMA immune-positive cells (arrows) are seen around central vein (CV), around blood sinusoids (S) (in 6e) & within the portal area (P) (in 6f). 6g&6h group III (Recovery group): Showing widely spread α-SMA immune-positive cells (arrows) around the central vein (CV), around the blood sinusoids (S) (in 6g) & around sinusoids (S) and within the portal area (P) (in 6h). 6i&6j group IV (Acetaminophen + Nanocurcumin group): Some α-SMA immune-positive cells (arrows) are noticed around the central vein (CV), around the blood sinusoids (S) (in 6i) & around sinusoids (S) and within the portal area (P) (in 6j) 6k&6l group V (Acetaminophen + MSCs group): Few α-SMA immune-positive cells (arrows) are demonstrated around the central vein (CV), around the blood sinusoids (S) (in 6k) & around sinusoids (S) and within the portal area (P) (in 6l) [α-SMA immunostaining, x200]. 6m: Histogram demonstrating the mean area percent of α-SMA positive reaction. [a as compared to group I, b as compared to group II, c as compared to group III & d as compared to group IV (highly significant difference at $P < 0.01$)]

## DISCUSSION

Overdose of paracetamol is a major common problem due to its common use, which may result from a defective awareness concerning its possible worse effects. Paracetamol can cause liver injury, possibly leading to failure[28]. However, cases often seek clinical advice after the start of first manifestations, usually twenty-four hours following paracetamol intake, which is delayed, & unfortunately death was followed 3 to 4 days after intoxication, and in some patients, it may occur after 14 days[29].

The rats of groups II & III exhibited reduced physical activity. This might be suggested by the paracetamol toxic effect causing liver injury, metabolic alterations as increased energy expenditure and protein catabolism resulting in muscle wasting.

Acute liver injury was induced in the current study by intraperitoneal injection of paracetamol. Most researchers use the intraperitoneal route of APAP, as it is more reproducible and easy handling. Moreover, intraperitoneal rather than oral route was recommended, as it was reported that liver injury is variable because of individual difference in absorption[30].

The enzymatic metabolism of xenobiotics & drugs into non-toxic compounds that accomplished in liver is fundamental for suitable body function[31]; the change of this condition results in displacement towards oxidants production, which bind to nuclear proteins or lipids causing mutations, membrane disruption, and altered enzymatic activity, leading to impaired liver function[32,33].

The highly significant rise in serum ALT in acetaminophen group verified the drug toxic effect causing oxidative stress and leading to disruption of hepatocytes membrane. This was supported by the highly significant elevation in MDA recorded in this group versus the control.

Enzymatic release from hepatocytes' cytoplasm into blood stream is synchronized with cell damage[34]. This was produced by N-acetyl-p-benzoquinone imine (NAPQI) effect, a metabolic product of paracetamol, which leads to lipid peroxidation of liver cells, followed by increase cell permeability and increase in ALT levels in blood[6,35]. This suggestion is concomitant with a previous paracetamol hepatotoxicity model which proved significant increase in serum liver enzymes and tissue MDA levels due to membrane phospholipid degeneration[17]. Accordingly, the current increase in oxidative stress might mediate the liver injury noticed in the present study.

Disruption of hepatocytes functions was confirmed biochemically in comparison to control rats as a highly significant reduction in albumin & disturbed protein synthesis. Additionally, a highly significant elevation of serum bilirubin that could be due to defective metabolism. This supposition agreed with former investigators who mentioned that the drop in serum albumin could be secondary to endoplasmic reticulum (ER) damage[36]. While hyperbilirubinemia was reported to be the outcome of lost cytochrome P450 function followed by impairment of hepatic bilirubin uptake and conjugation[37].

Disorganized hepatic lobular architecture as well as the appearance of cytoplasmic vacuolations and deeply stained nuclei in some hepatocytes were demonstrated in paracetamol group. This could be the result of the toxic effect of paracetamol metabolites leading to hepatocytes necrosis and accumulation of lipid droplets. These morphological alterations were similarly recorded in mice and rats 12 and 24 hours, respectively following paracetamol administration[17,34].

The large doses of paracetamol could lead to massive production of NAPQI that accumulate in liver cells, become inactive when conjugated with glutathione (GSH) and combined with different proteins producing adducts of protein. This was followed by depletion of glutathione & adenosine triphosphate (ATP), and dysfunction of mitochondria[38]. As well as it resulted in disruption of calcium balance which leads to stimulation of ATPases, proteases, phospholipases, & endonucleases ending in membrane damage[39], oxidative stress, & disrupted liver cytoarchitecture and necrotic cell death[40].

Therefore, cytoplasmic vacuolations in hepatocytes could be the consequence of fat droplets depositions secondary to paracetamol that induced disturbance in the phospholipid metabolism[41]. This was similarly reported in another work where they suggested that the only liver cells noticed to develop necrosis, evidenced by vacuolation and pyknotic nuclei, were those containing paracetamol protein adducts[42].

In the current study, paracetamol-induced hepatotoxicity was accompanied by an enhanced inflammatory response (congestion & inflammatory infiltration). These were like those described in other mouse studies 12 hours[34] and 24 hours after APAP administration[43]. Necrotic hepatocytes might be the cause of inflammation releasing damage-associated molecular patterns that stimulate Kupffer cells receptors with subsequent production of inflammation mediators [as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) & IL-1β], reactive oxygen species and chemokines, thus upregulating pro-inflammatory signals. These pro-inflammatory factors enhance inflammatory cells recruitment primarily neutrophils and monocytes into the necrotic hepatic areas to eliminate cell debris and promote the inflammatory process[44,45]. Such inflammatory cell infiltration is a source of oxidative stress[46], forming superoxide anion by nicotinamide adenine dinucleotide phosphate oxidase causing production of hydrogen peroxide and other oxidative agents resulting in direct cellular toxicity[38].

Several studies indicated that HSCs are the most important profibrogenic cells in hepatic tissue where they are transformed to about 82–96% of all myofibroblasts in toxic, cholestatic & fatty liver models[47]. GFAP is the authentic marker for hepatic stellate cells[48]. An increase in

numbers of HSCs expressing GFAP following liver injury is an indicator of local proliferation and accumulation of cells (by chemotaxis) in regions of injury[49]. In addition, the induction of α-SMA is considered an index of HSCs stimulation[48]. The α-SMA expressing myofibroblasts can produce fibrogenic cytokines and collagen, that contribute to fibrosis[50]. This could explain the non-significant boost in area percent of collagen fibers in group II versus group I based on the non-significant elevation in GFAP positive HSCs & α-SMA positive myofibroblasts in group II versus group I.

By day 15 (group III), failure of hepatic regeneration was illustrated biochemically by the continuous rise of serum ALT & bilirubin and liver MDA with highly significant decrease in serum albumin versus group II. Histologically by the appearance of disorganized hepatic cords, ballooning of most hepatocytes, with severely vacuolated cytoplasm and pyknotic nuclei. This could be clarified by liver regeneration next to paracetamol toxicity was dose-dependent and liver repair might be repressed following high paracetamol overdose causing progression of ALI to acute liver failure[51]. Further support came from another study done on mice where the high overdose of acetaminophen was followed by suppression of hepatic regeneration[52].

These results could be explained by APAP toxicity that induced an intense enhancement of genes of glutathione metabolism in hepatocytes with subsequent cellular stress in hepatocytes. Thus, the depleted GSH caused a delayed recovery and is considered an essential intermediate phase of hepatocyte's necrosis[53].

Although the main goal of immune response was necrotic debris removal and start of regeneration, an aggravation of existing tissue injury has occurred[54]. This could be explained based on the fact that inflammatory response includes three stages; vascular phase, cellular phase (migration of inflammatory cells) which are both followed by resolution phase. In case of failure of resolution, extended tissue destruction was followed by healing by fibrosis[55]. Additionally, it was reported that necrotic liver cells, endothelial and Kupffer cells secrete transforming growth factor-beta 1 (TGF-β1) which triggered trans-differentiation of quiescent HSCs into myofibroblasts[56] with subsequent formation of extracellular matrix (ECM) promoting fibrosis in liver[57].

In the current work group III showed regression of inflammatory infiltration, while congested portal vein and blood sinusoids were noticed. This was aggravated by collagen fiber deposition due to activated HSCs that trans-differentiate to multiple myofibroblast starting a process of fibrogenesis. Further confirmation was complemented statistically by a highly significant rise in area percent of collagen deposition, GFAP & α-SMA immunoreactivity versus group II. Collagen fibers deposition in area of affected hepatocytes was explained by increased oxidative stress parameter MDA with consequent HSCs activation. In other studies, some authors had attributed the presence of some fibrosis to be secondary to cell ballooning degeneration and was usually demonstrated encircling hepatocytes[58]. In addition, activated HSCs also produce inhibitors of interstitial collagenases. Thus, hinder collagen degradation resulting in an accumulation of ECM[59]. Respectively, the collagen fiber deposition disrupts the normal liver architecture by constituting a network of fibrous barriers that lead to continuous and progressive deterioration of the liver metabolic and detoxifying functions[60]. The previous findings are consistent with the results of hepatotoxicity rat model after 15 days of single intraperitoneal injection of acetaminophen[61].

Nanoparticles are an emerging field which can overcome difficulties that face conventional medicine by increasing curcumin's biological activity, solubility, bioavailability and long-time in circulation[62]. In addition, administration of curcumin as nanoparticles could easily pass through sinusoidal fenestrae and evade capture by hepatic macrophage (von Kupffer cells) and thus targeting hepatic stellate cells and hepatocytes[63,64]. Thus, curcumin nanoparticle was tested in ALI model in the present work.

The nanocurcumin treated rats (group IV) exhibited a highly significant reduction in values of ALT and bilirubin, & a highly significant elevation of albumin than group III and a highly significant difference versus group I. This could be suggested by the ability of curcumin nanoparticles to stabilize hepatocyte's structure, protein synthesis and improvement of bilirubin metabolism leading to proper excretion through its antioxidant and anti-inflammatory effect. This was reflected biochemically by a highly significant drop in oxidative stress marker MDA, a lipid peroxidation marker. Besides, the normal appearance of some hepatocytes with vesicular nuclei and regression of vascular congestion and inflammation while other hepatocytes still exhibited pyknotic nuclei and cytoplasmic vacuolations.

These preceding findings coincide with prior study that administrated nanocurcumin for fourteen days in rat model of ALI produced by cisplatin[18]. Further researchers examined the use of nanocurcumin for 2 weeks in a CCL4 hepatotoxic model and recorded an improvement in liver enzymes, MDA levels in addition to amelioration of fatty degeneration, leukocyte infiltration, and hepatocyte necrosis[65].

Curcumin nanoparticles, being an immunomodulatory and anti-inflammatory agent could inhibit the inflammatory cellular proliferation and differentiation, as well as result in reduction of multiple inflammatory cytokines, including interleukin IL1-β and IL-6[66]. This agreed with our suggestion explaining the nanocurcumin's role in reduction of vascular congestion and inflammatory cell infiltration. This could be also mediated by the ability of nanocurcumin to potentiate the antioxidant system in the affected liver by rising the intracellular GSH & superoxide dismutase (SOD) levels and alleviating lipid peroxidation induced by MDA[67].

Nanocurcumin group (group IV) demonstrated a highly significant reduction in collagen deposition versus recovery group while a highly significant rising was recorded versus the control. Resonating with the decreased collagen deposition, GFAP and α-SMA area percent showed a highly significant drop versus group III and a highly significant rise when compared to group I. This might be related to antifibrotic property of nanocurcumin suppressing HSCs proliferation, decreasing TGF-β, HSCs trans-differentiation into myofibroblasts and collagen deposition[68]. These findings agree with that of former trials where nanocurcumin recorded decreased collagen deposition in aluminum oxide and cadmium hepatotoxicity models[69,70]. Furthermore, another postulated mechanism of the antifibrotic effect of curcumin was achieved by inhibiting the chemokine and cytokines genes expression released from Kupper cells [mitogen platelet derived growth factor, TNF-α & IL-6] which were directly related to fibrosis that stimulate HSCs proliferation and activation[71,72,73].

In the current work (group V), MSCs were administered intravenously through the rat tail vein after induction of ALI, based on former documents that showed better effectiveness of intravenous route of MSCs injection than intraperitoneal, intrasplenic and intrahepatic routes in decreasing hepatic inflammatory factors (IL-1 β, IL-6, and TNF-α), restoring hepatic function, reversing fibrosis, and rescuing liver failure[74,75]. Additionally, it has been documented that treatment with MSCs showed mild effective influence in decompensated cirrhotic liver (chronic lesion), whereas in acute liver failure, MSCs therapy has superior impact especially with its early use[76]. Therefore, in the current study MSCs injection was early at day 2 of the experiment.

Upon injection, MSCs migrate to the injured area. The surge of inflammatory cytokine in areas of inflammation stimulates stem cells migration to injured tissues[77]. This was detected in the current study where MSCs homed at the site of liver injury 3 days after their administration as previously stated[20].

Administration of MSCs (group V) revealed a highly significant decrease in values of ALT& bilirubin, and a highly significant increase in albumin values versus group III, while a highly significant difference was illustrated compared to group I. This might be assumed by the therapeutic action of MSCs on hepatic cells through secretion of trophic factors which restore hepatocyte integrity and endocrine function as well stem cells could improve hepatic uptake and conjugation of bilirubin. These results are in harmony with a former study which observed improvement in liver function index and hepatocytes degeneration following 2 weeks of MSCs treatment of CCL4 induced-liver toxicity in mice[78].

Correlating with recorded liver function results, tissue MDA in group V showed a highly significant reduction than group III and highly significant increase than group I. This could support the demonstrated nearly well-organized hepatic lobule architecture with apparently normal hepatocytes, in addition to the relieved vascular congestion and inflammation. This coincides with a previous report that found diminished hepatocellular injury, and reduction in macrophage infiltration & inflammatory factors following stem cells administration[78]. Moreover, it was suggested that homed MSCs start their effect by secreting growth factors that contribute to liver regeneration through stimulation of cell proliferation, enhancement of angiogenesis, and suppression of apoptosis[79,80,81]. Furthermore, MSCs promote liver repair by its immunomodulatory effect through release of IL10, nitric oxide or prostaglandin E2. This results in T-cells downregulation, B-cells inhibition. Additionally, MSCs have immunosuppressive mechanisms mediated by cytokines (as IL1β, TNF-α, or interferon gamma (IFN-γ)[15,82,83].

Similarly other investigators related the improvement hepatic alterations to the antioxidant power of MSCs that were shown after one week of stem cell administration in acetaminophen induced liver toxicity[84]. MSCs upregulate superoxide dismutase expression as an antioxidant response, hence stimulating antioxidant and cytoprotective activity and subsequently decrease hepatocyte cell death[85,86].

Regarding the role of MSCs on collagen deposition and liver fibrogenesis, stem cell treated animals showed a highly significant diminution in area percent of collagen fibers, GFAP & α-SMA immunopositivity than recovery group & a highly significant increase versus group I. These results are formerly reported where MSCs markedly decreased collagen deposition in ALI model[61]. Moreover, it was found that coculturing of MSCs with hepatic stellate cells, HSCs underwent apoptosis, and reduced their production of ECM components, which in turn prevented progression of fibrosis and played an important role in alleviating liver diseases[87].

Additionally, Yao *et al*., (2021)[77] observed a reduction in α-SMA expression in MSCs treatment group after CCL4 induced liver fibrosis. Further support of the antifibrotic effect of MSCs was confirmed by the recorded decrease in GFAP and α-SMA expression after MSCs administration in rat cirrhotic liver model[88]. MSCs could directly and indirectly inhibit HSCs proliferation and activation, and in turn suppress collagen production. The direct suppression was through arresting HSCs in G0/G1 phase[89]. Indirectly, secretomes released from MSCs include various growth factors and mediators like hepatocyte growth factor and IL-10, that either inhibited HSCs proliferation or promoted HSCs apoptosis and eventually can attenuate liver fibrosis[89,90].

Thus, MSCs can enhance liver repair by preventing hepatocytes apoptosis and reducing inflammatory & fibrogenic cytokine[91]. Added to that, stem cells administration could reduce collagen deposition through the inhibition of TGF-β1 & α-SMA expression[92]. Prior

investigators also linked MSCs antifibrosis effect to their ability of degradation of excess ECM either directly by production of matrix metalloproteinases (MMP), or indirectly, by activation of immune cells to increase their production of MMP and inhibit the release of their inhibitors as tissue-induced metalloproteinase inhibitor (TIMPI)[93,94].

Both MSCs or nano-curcumin ameliorated acetaminophen induced liver toxicity however MSCs showed potentiated effect than nano-curcumin. Comparing MSCs with nano curcumin treated ALI rats in the sitting work, there was a highly significant decrease in serum levels of ALT, bilirubin, and tissue MDA in addition to a highly significant increase in serum albumin level. Along with the highly significant reduction in collagen fiber deposition, GFAP and α-SMA positive immunoreactivity in the corresponding immune-stained liver section. This could indicate a better regenerative effect of MSCs than nanocurcumin. Such suggestion might be based on the therapeutic potentials of MSCs that could be mediated by their secretion of growth factors, chemokines and cytokines that played an important role in injury amelioration. Additionally, MSCs could exert their effects even if they were not differentiated to tissue-specific cells. This occurred via their paracrine roles as immunomodulatory, trophic "nurturing" and anti-scarring effects. Such advantages might increase the range of stem cells therapeutic applications[61].

## CONCLUSION

Although paracetamol is considered a relatively safe medication, non-intentional overdose may result in impaired liver functions, oxidative stress, inflammation, cellular necrosis, and eventually hepatic fibrosis. The present work could provide evidence for the therapeutic impact of curcumin nanoparticles and MSCs on acetaminophen-induced ALI in rats. However, MSCs exhibited more pronounced effects on restoring biochemical functions and histological structure compared to nanocurcumin.

## RECOMMENDATIONS

It is recommended to study the potential effect of higher doses and / or longer duration of MSCs and curcumin nanoparticles. However, it is substantial to explore long term side effects of MSCs and the probable toxicity of natural product- nanoparticles especially when utilized for the treatment of chronic disorders. Further extension of the experimental study for more than one month is required to detect whether the fibrosis will show more deterioration or improvement.

## CONFLICT OF INTERESTS

There are no conflicts of interest.

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**الملخص العربى**

# دراسات هستولوجية وهستوكيميائية مناعية على تأثير الكركم المحمل على جزيئات النانو مقابل الخلايا الجذعية الوسيطة على الإصابة الحادة بالكبد المحدثة بالاسيتامينوفين في ذكور الجرذان البيضاء البالغة

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**الخلفية:** تعد الإصابة الكبدية الحادة بالأسيتامينوفين مشكلة شائعة وقد تسبب الإصابة بفشل الكبد الحاد. يمتلك الكركم خصائص مضادة للأكسدة ومضادة للالتهاب ومضادة للتليف و لكن ضعف ذوبانه وأيضه السريع يجعلان تأثيرَه العلاجيَ محدودًا. قد تتغلب تركيبة النانو على هذا الضعف وتمكن من تحسين خصائصه. حظي العلاج بالخلايا الجذعية الوسيطة باهتمام بتسجيلها تأثيرات متعددة الاتجاهات لإصلاح الأنسجة وتجديدها في العديد من الأبحاث. وذلك من خلال قدرتها على تثبيط الإجهاد التأكسدي والالتهاب والتليف بالإضافة إلى تأثيرها الغذائي نظيرُ الصَّمَّاوِيّ.

**الهدف من العمل:** مقارنة التأثير العلاجي المحتمل للكركم المحمل على جزيئات النانو مقابل الخلايا الجذعية الوسيطة على الإصابة الحادة بالكبد المحدثة بالاسيتامينوفين في نموذج الجرذ

**المواد وطرق البحث:** تم تقسيم أربعة وثلاثون من ذكور الجرذان البيضاء التى تبلغ ثلاثة اشهر من العمر إلى المجموعة المانحة، المجموعة الاولى (الضابطة) ، المجموعة الثانية (الاسيتامينوفين) ، المجموعة الثالثة (التعافي)، المجموعة الرابعة (الاسيتامينوفين والنانو كركم)، والمجموعة الخامسة (الاسيتامينوفين و الخلايا الجذعية الوسيطة). في نهاية مدة التجربة تم إجراء التحاليل الكيميائية الحيوية ] لناقل أمين الألانين والأَلْبومين والبِيلِيرُوبِين في المَصْل ، والمالوندياالدهيد في الانسجة[، الدراسات الهستولوجية والهستوكيميائية المناعية [للحمض الليفي الدبقي ولأكتين العضلات الملساء ألفا] والتقييم الإحصائي.

**النتائج:** سجلت مجموعة الاسيتامينوفين ارتفاعات ذات دلالة احصائية فى مستويات ناقل أمين الألانين والبِيلِيرُوبِين، والمالونديالدهيد وانخفاض فى مستوى الأَلْبومين . بينما كانت هناك زيادة بدون دلالة احصائية فى ترسب الياف الكولاجين وكميه التفاعل المناعي للحمض الليفي الدبقي ولأكتين العضلات الملساء ألفا مقابل المجموعة الضابطة. كما لوحظت فصوص كبدية غير منتظمة البنيان وبعض الخلايا الكبدية ذات فجوات وأنوية منكمشة داكنة الصبغة وتغلغل للخلايا الالتهابية. أظهرت مجموعة التعافي تفاقمًا ملحوظًا للنتائج المذكورة سابقًا. ، بينما حسن العلاج باستخدام الكركم المحمل على جزيئات النانو والخلايا الجذعية الوسيطة من إصابة خلايا الكبد والإجهاد التأكسدي والالتهاب والتليف مع تميز الخلايا الجذعية الوسيطة.

**الاستنتاج:** كان للعلاج بكل من الكركم المحمل على جزيئات النانو والخلايا الجذعية الوسيطة تأثير علاجي ضد السمية الكبدية بأسيتامينوفين. ومع ذلك، أظهر علاج الخلايا الجذعية الوسيطة تأثيرات أفضل على استعادة بنية خلايا الكبد ووظيفتها.