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SELENIUM NANOPARTICLES ATTENUATE HIGH-FAT DIET-INDUCED NAFLD IN MALE ALBINO RATS WITH AND WITHOUT DIETARY MODIFICATION: HISTOPATHOLOGICAL AND BIOCHEMICAL EVALUATION

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic hepatic disorder, yet no pharmacological therapies have been approved to prevent or treat this condition. This study evaluated the potential therapeutic effects of selenium nanoparticles (SeNPs) in a high-fat diet (HFD)-induced NAFLD model in adult male albino rats, with or without dietary modification. Forty-two rats were randomly divided into four groups. Group I received standard chow, with one subgroup receiving vehicle and the other SeNPs (2 mg/kg/day) during weeks 8–10. Group II was fed an HFD, with one subgroup used for model confirmation at 8 weeks and the other maintained on HFD for 10 weeks. Group III received SeNPs during weeks 8–10 while either continuing HFD or switching to standard chow. Group IV underwent dietary modification alone. HFD administration induced marked hepatic steatosis, hepatocellular injury, and fibrosis, accompanied by significant elevations in serum ALT, AST, total cholesterol, LDL, triglycerides, fasting blood glucose, fasting insulin, and HOMA-IR. SeNPs treatment significantly attenuated these alterations, with the greatest improvement observed when

combined with dietary modification. Histological and ultrastructural changes were markedly improved, and trichrome staining demonstrated reduced fibrosis. SeNPs treatment significantly reduced tissue malondialdehyde levels while increasing superoxide dismutase and total antioxidant capacity. HFD-induced increases in SMA and TGF- β expression were also significantly attenuated by SeNPs, particularly when combined with dietary modification. In contrast, spontaneous dietary recovery produced only partial improvement. These findings indicate that SeNPs attenuate HFD-induced steatosis, oxidative stress, metabolic disturbances, and fibrogenic changes, with enhanced effects when combined with dietary modification.

Keywords: High fat diet; NAFLD; liver; selenium nanoparticles.

1. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has emerged as the most prevalent chronic hepatic disorder worldwide, yet no pharmacological therapies have been approved to prevent or treat this condition.^[1] Its prevalence varies according to age, gender, ethnicity, and diagnostic methods, with a global average estimated at 24.1%, ranging from 13.5% in Africa to 31.8% in the Middle East.^[2, 3]

NAFLD encompasses a spectrum of conditions, beginning with simple hepatic steatosis and potentially progressing to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, end-stage liver failure, and hepatocellular carcinoma.^[4] It is defined as ectopic lipid accumulation in the liver in the absence of excessive alcohol intake or other attributable cause.¹ Accumulation of more than 5% fat, mainly in the form of triglycerides (TGs), is the minimal requirement for the histological diagnosis of steatosis.^[4]

Oxidative stress is considered a key contributor to the pathogenesis and progression of NAFLD.^[1,4] Excessive hepatic lipid accumulation increases the generation of reactive oxygen species (ROS), disrupting the balance between oxidant production and endogenous antioxidant defenses. This excess ROS promotes lipid peroxidation, mitochondrial dysfunction, and hepatocellular injury, while also activating inflammatory and fibrogenic pathways that drive disease progression from simple steatosis to steatohepatitis and fibrosis.^[1, 4] Accordingly, enhancing endogenous antioxidant defenses and attenuating oxidative stress have emerged as promising therapeutic strategies for limiting hepatic injury in NAFLD.

Selenium (Se), an essential trace mineral, is required for the synthesis of antioxidant enzymes and selenoproteins that support DNA synthesis and cellular defense against oxidative damage.^[5] Despite these benefits, Se has a narrow therapeutic window, and excessive intake of inorganic or organic Se-based compounds frequently results in toxicity.^[6] Recently, selenium nanoparticles (SeNPs) have attracted attention as an alternative formulation offering lower

toxicity, higher biocompatibility, and comparable therapeutic potential, with demonstrated benefits in diabetes, bone toxicity, colitis, and drug-induced toxicity.^[6, 7] However, the potential hepatoprotective effects of SeNPs in diet-induced NAFLD, particularly in relation to dietary modification, remain insufficiently investigated.

Dietary modification and lifestyle intervention remain the cornerstone of NAFLD management, but adherence and long-term efficacy are often variable,^[1] creating interest in adjunctive pharmacological or nutraceutical approaches such as SeNPs.

The present study investigates the potential hepatoprotective effects of SeNPs as antioxidant agents in a rat model of high-fat diet-induced hepatic steatosis, with and without dietary modification, using both histopathological and biochemical evaluations.

2. MATERIALS AND METHODS

2.1 Chemicals

2.1.1 Selenium nanoparticles preparation

SeNPs were prepared by reducing selenite according to Zhang et al.^[8] With limited modification. Briefly, 1 g of Chitosan and 0.8 g of Vitamin C were completely dissolved in 100 mL of 1% (w/w) acetic acid to attain CHITOSAN/Vitamin C solution. After that, 5 mL of selenite aqueous solution containing 0.2 g of sodium selenite was added to the Chitosan/Vitamin C solution and vigorously stirred (500–600 rpm).^[9]

2.2 In-Vitro Characterization of SeNPs

SeNPs particle size and polydispersity index (PDI) were evaluated using dynamic light scattering (Zetasizer[®] Nano ZS series DTS 1060, Malvern Instruments S.A, Worcestershire, UK). Zeta potential was also measured at 25 °C using a cell voltage of 150 V.

Morphological examination using transmission electron microscope (TEM) was performed on Talos F200i high-resolution transmission electron microscope at an accelerating voltage of 200 kV. Samples for TEM were prepared by placing a droplet of colloid suspension

in the respective solvent on a Formvar carbon-coated, 300-mesh copper grid (Ted Pella) and allowing them to evaporate in air at ambient conditions. Size distribution and average size were determined using an image analysis software package. Micrographs were captured at a magnification of $\times 74,000$.

2.2.1 Experimental animals

Forty-two adult male albino rats, weighing 150–200 g and aged 6–8 weeks, were obtained from the Animal House of the Physiology Department, Faculty of Medicine, Alexandria University. Animals were housed under standard laboratory conditions, including controlled temperature, humidity, and a 12-hour light/dark cycle. All experimental procedures were conducted in accordance with the research ethics guidelines approved by the Research Ethics Committee, Alexandria Faculty of Medicine (IRB No. 00012098, FWA No. 00018699, Approval No. 0107327).

2.2.2 Induction of NAFLD model

Animals were fed an in-house prepared high-fat diet (HFD) providing 58% of total calories as fat formulated to contain 20 g fat/100 g diet. ^[10] (Table 1)

Table (1): Components of high fat diet.

Component	(Gram %)
Casein	19.6
Corn starch	36.24
Sucrose	10.75
Cellulose	7
Butter oil	19
Soybean	1
Vitamin mix	1.27
L-cystine	0.25
Acetyl choline	0.27
Cholesterol	0.59

2.2.3 Experimental design

2.2.3.1 Animals were randomly allocated into four experimental groups:

Group I (Control, n = 12)

- **Group IA:** standard chow + vehicle
- **Group IB:** standard chow, with SeNPs administered from week 8 to week 10

Group II (HFD, n = 12)

- **Group IIA (HFD, 8 weeks, n = 6):** HFD for 8 weeks, used for model confirmation.
- **Group IIB (HFD, 10 weeks, n = 6):** HFD continued for 10 weeks (disease progression control).

Group III (SeNP-treated, n = 12): rats received selenium nanoparticles (SeNPs), 2 mg/kg body weight, via daily oral gavage from week 8 to week 10.^[11]

- **Group IIIA (SeNPs + HFD, n = 6):** continued HFD for 10 weeks, with SeNPs from week 8 to week 10
- **Group IIIB (SeNPs + diet modification, n = 6):** switched to standard chow from week 8 to week 10, with concurrent SeNPs

Group IV (Spontaneous recovery, n = 6): switched to standard chow from week 8 to week 10, without SeNPs (diet modification only)

2.2.3.2 Assessment of the experimental animals

The mortality rate of rats was recorded during the duration of the experiment. Body weights of rats were recorded at the beginning of the experiment and weekly throughout the study period. The obtained data was used to calculate the percentage change in the body weight of the rats as $(\text{final weight} - \text{initial weight}) / \text{initial weight} \times 100$.^[12]

2.3 Histological assessment

The liver was excised and divided into four portions for comprehensive histological and ultrastructural evaluation:

1. **Paraffin sections** the first portion was fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Sections of 4.5 μm thickness were cut and stained with Hematoxylin and Eosin (H&E) for general histological assessment, and trichrome stain for evaluation of fibrosis.
2. **Osmium tetroxide staining** the second portion was cut into ~ 2 mm pieces and immediately immersed in osmium tetroxide. Sections were prepared and examined under a light microscope to visualize lipid deposits.
3. **Oil Red O (ORO) staining** the third portion was dehydrated, flash-frozen with dry ice, and sectioned at 10 μm thickness using a cryostat. Frozen sections were stained with Oil Red O following conventional protocols, counterstained with Hematoxylin, and examined under a light microscope to assess neutral lipid accumulation.
4. **Transmission electron microscopy (TEM)** The fourth portion was cut into ~ 1 mm³ pieces, fixed in 3% phosphate-buffered glutaraldehyde, and processed to obtain ultrathin sections. These were examined using transmission electron microscopy to assess hepatocellular ultrastructure.

Two blinded observers performed the histological assessment according to Kleiner et al, histopathological examination for assessing NAFLD Activity Score (NAS) and Fibrosis staging was also done.^[13, 14]

2.4 Morphometric analysis

Digital images were obtained from ORO, Osmium tetroxide, and trichrome-stained sections using a digital camera connected to the microscope (n=6 per group). Measurement was expressed in the form of a percentage area using NIH Image J software.

2.5 Biochemical assessment

Twenty-four hours after the last dose, twelve hours after the last meal, blood samples were collected from the retro-orbital plexus in plain tube vacutainers and allowed to stand for 30 minutes to clot at room temperature, then centrifuged, and the sera were aliquoted to be kept at -20 °C for the following chemical analysis:

1. liver function: serum glutamic-pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) using colorimetric assays Spectrum Diagnostics (Egypt).^[15]
2. Fasting plasma glucose (FPG) using colorimetric assays, fasting insulin (F. insulin) using ELISA kit FineTest China Cat.#EH0374, and homeostasis model assessment of insulin resistance (HOMA IR).^[16, 17]
3. Lipid profile: plasma triglyceride, total cholesterol level, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) using colorimetric assay method by Spectrum Diagnostics (Egypt).^[15]
4. Oxidative markers: Malondialdehyde (MDA) and Superoxide dismutase (SOD) assessed using calorimetric methods by Sigma-Aldrich, Germany. Cat. #MAK085 and BioAssay systems Hayward, USA Cat#ESOD-100 respectively

Total antioxidant capacity (TAC) in tissue was analyzed using calorimetric methods by Abcam united kingdom # ab65329.^[18]

2.6. Quantitative RT-PCR analysis of TGF- β and α -SMA Transcripts

2.6.1 Total RNA Isolation and Reverse Transcription

a) RNA extraction and cDNA synthesis

Total RNA was extracted following the instructions provided by RNeasy Mini Kit (Qiagen, Hilden, Germany). The isolated RNA was resuspended in RNase-free Diethylpyro-carbonate (DEPC)- treated water and was then stored at -80 °C. RNA quality was

spectrophotometrically determined at 260/280 nm, pure preparations were considered when RNA ratios were around 2.0. RNA All the RNA handling and preparation steps were performed under strictly sterile and RNase-free conditions. After that, a complementary deoxyribonucleic acid (cDNA) was synthesized using 100 ng of total RNA, random hexamer primer and superscript II reverse transcriptase (RT) (Invitrogen, USA).

b) Gene expression analysis by quantitative RT-PCR:

Quantitative polymerase-chain reaction (qPCR) was performed in One Step real-time PCR system (Applied Biosystems, USA). Amplification of the synthesized cDNA was performed in duplicates in a 25 µl reaction volume containing 1× SYBR® Green PCR Master Mix with pre-mixed ROX dye as a passive reference (Applied Biosystems, USA). The specific primer pair for Collagen: sense primer: 5'-GACATGTTCAGCTTTGTGGACCTC-3' and antisense primer: 5'-GGGACCCTTAGGCCATTGTGTA-3', TGF-β: sense primer; 5'-CTACTACGCCAAAGAAGTCACC - 3' and antisense primer; 5'-GAAAGCCCTGTATTCCGTCTC - 3'. Moreover, the specific primer pair for α-SMA: sense primer; 5'-CACCATCGGGAATGAACGCT - 3', antisense primer; 5'-CGAGAGGACGTTGTTAGCATAGAG-3' and the GAPDH: sense primer; 5' – AACGGGAAACCCATCACCA-3' and antisense primer; 5' - CGGAGATGATGACCCTTTTG-3'. The amplification for cDNA consisted of one cycle at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 30 s, a 60 °C annealing step for 30 s, and an extension step at 72 °C for 30 s. Melting curve analysis of products was routinely performed after amplification using an incremental temperature increase from 60 °C to 95 °C where the identity of the PCR product was confirmed. A negative control without cDNA was run with every PCR to assess the specificity of the reaction. Data analysis was performed using StepOne™ Software v2.3 where the level of expression of were determined by the comparative CT method for gene expression relative to the housekeeping gene glyceraldehyde- 3 phosphate dehydrogenase (GAPDH).^[19]

2.7 Statistical analysis

Data was analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Shapiro-Wilk test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean and standard deviation. Significance of the results obtained was judged at the 5% level. One-way (ANOVA) test was used for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons.

3. RESULTS

3.1 Characterization of SeNPs

SeNPs were spherical with a uniform size distribution (65 ± 10 nm) as shown by TEM (**Figure 1A**). UV–Vis spectroscopy showed a characteristic surface plasmon resonance peak at approximately 258 nm, confirming SeNPs. Chitosan-coated SeNPs exhibited a positive zeta potential (+38 mV), indicating high stability. DLS showed a larger hydrodynamic size (258.4 nm) and a moderate, homogeneous polydispersity (PDI 0.1–0.4) (**Figure 1B**).

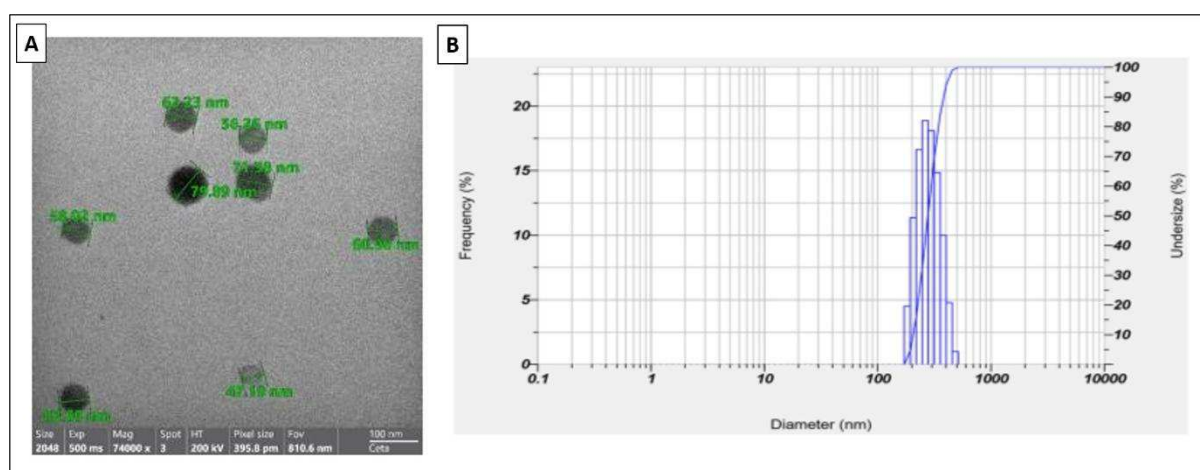


Fig. 1: Invitro characterization of SeNPs: (A) Morphology and size of SeNPs using TEM, scale bar= 100 nm and (B) Particle size distribution.

3.2 Assessment of experimental animals

No mortality was recorded in rats across all groups during the study period. A significant increase in body weight was observed in all experimental groups compared to the control ($p < 0.001$). The HFD group at 10 weeks exhibited the highest percentage increase (mean = 38.09%), followed by the spontaneous recovery group (36.82%). While the (SeNPs + HFD) subgroup showed moderate weight gain (33.01%), lower than the untreated HFD groups, the (SeNPs + normal diet) subgroup demonstrated the lowest increase (26.70%). This suggests a potential preventive effect of SeNPs against diet-induced obesity, particularly when combined with dietary modification (Table 2).

Table (2): Comparison between the different studied groups according to % increase in body weight

	Control (n = 6)	HFD		Treated		Spontaneous recovery (n = 6)	F	p
		8w (n = 6)	HFD 10w (n = 6)	(SeNPs+ HFD) (n = 6)	(SeNPs+ normal diet) (n = 6)			
% increase in body weight								
Min.	5.55	26.66	33.33	28.88	22.70	32.22	77.156*	<0.001*
Max.	7.14	34.28	42.85	37.14	30.70	41.42		
Mean	6.35	30.47 ^a	38.09 ^{ab}	33.01 ^a	26.70 ^{acd}	36.82 ^{abe}		
± SD.	0.66	3.16	3.95	3.43	3.32	3.82		
p ₁		<0.001 [*]	<0.001 [*]	<0.001 [*]	<0.001 [*]	<0.001 [*]		
p ₂			0.004 [*]	0.753	0.361	0.022 [*]		
p ₃				0.103	<0.001 [*]	0.983		
p ₄					0.024 [*]	0.350		
p ₅						<0.001 [*]		

6 replica for each group

SD: Standard deviation

F: F for One way ANOVA test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Tukey)

p: p value for comparing between the different studied groups, p₁: p value for comparing between Control and each other group, p₂: p value for comparing between HFD (8w) and each other group

p₃: p value for comparing between HFD (10w) and each other group, p₄: p value for comparing between SeNPs+HFD and each other group, p₅: p value for comparing between SeNPs+normal diet and each other group

*: Statistically significant at $p \leq 0.05$

a: Significant with Control, b: Significant with HFD (8w)

c: Significant with HFD (10w), d: Significant with SeNPs+HFD

e: Significant with SeNPs+normal diet

3.3 Histological studies

3.3.1 H & E staining

Light microscopic examination of the H&E-stained sections of both control groups (group IA & IB) showed normal hepatic architecture; hepatocytes appeared polyhedral in shape with granular eosinophilic cytoplasm and rounded, centrally located vesicular nuclei, arranged as branching and anastomosing cords radiating from the central vein. The hepatic sinusoids were lined by endothelial cells and Kupffer cells. At the corners of each lobule. The portal areas were seen. Each portal tract showed a branch of the hepatic artery, a branch of the portal vein, and a bile duct radicle enclosed by a scanty amount of connective tissue (**Figure 2 A & B**).

Following 8 weeks of a high-fat diet (HFD), a well-established and pronounced NAFLD model was confirmed, indicating successful model induction (**Figures 2 C & D**). Both high fat diet groups HFD for 8- and 10-weeks (group IIA and IIB respectively) showed the loss of normal hepatic architecture which was more pronounced in the 10-week group (**Figure 2 E & F**). Most of the hepatocytes appeared foamy with vacuolated cytoplasm and dark nuclei. Others appeared swollen with a single large cytoplasmic vacuole and peripheral nuclei (macro-steatosis). Blood sinusoids between the hepatocytes were obliterated. Several hepatocytes appeared ballooned with loss of nuclei with cytoplasmic perinuclear acidophilic clumps inside some hepatocytes (Mallory-Denk bodies). (NAS scoring system subgroup II A scored: 5 while subgroup II B scored: 8. Appearance of connective tissue septa was seen in both groups, with more extensive porto-portal fibrous septa and pseudo-lobule formation in the 10-week group (fibrosis stage F2 and F3, respectively). (**Figure 2 C, D, E & F**)

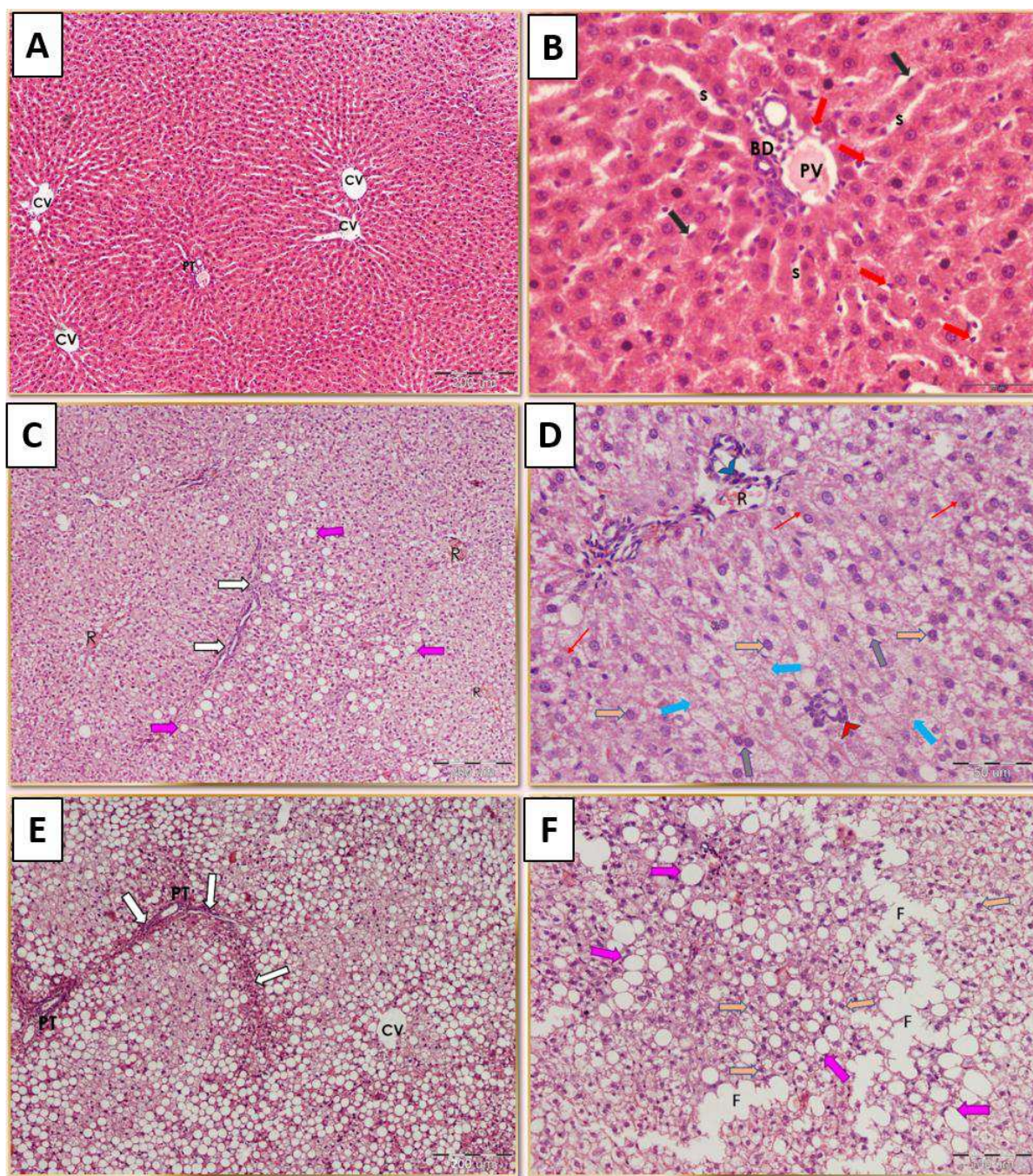


Fig. 2: Histological assessment of the effect of high fat diet on the liver: **A, B control Subgroups:** all control subgroups showed the same histological picture of the classical pattern of the hepatic architecture. Hepatocytes were arranged as branching and anastomosing cords radiating from the central vein. Sinusoids (s) are lined by endothelial cells (arrows) and Kupffer cells (red arrows). The hepatocytes appeared polyhedral in shape with granular eosinophilic cytoplasm and rounded, centrally located vesicular nuclei. The Portal areas were seen at the angles of the lobules. Each portal tract showed a branch of the hepatic artery, a branch of the portal vein (PV), and a bile duct (BD) radicle enclosed by a scanty amount of

connective tissue. **C & D HFD for 8 weeks:** Showing the loss of normal hepatic architecture of the radiating hepatic cords. Most of the hepatocytes appear foamy with vacuolated cytoplasm and dark nuclei. Some hepatocytes appear swollen with a single large cytoplasmic vacuole and peripheral nuclei (pink arrow). Blood sinusoids between the hepatocytes are obliterated. Some nuclei appear central (orange arrow), while others are eccentric (gray arrow). Other hepatocytes appear ballooned without nuclei (blue arrow). Note the cytoplasmic perinuclear acidophilic clumps inside some hepatocytes (thin red arrow). Mononuclear cellular infiltration was seen around the portal tract hyperplasia (white thick arrow) and in between hepatocytes (red arrowhead). R; extravasated RBCs. **E & F: HFD for 10 weeks:** showed massive hepatic affection with loss of the normal hepatic architecture where Note that most hepatocytes appear swollen with peripheral flattened nuclei. Numerous connective tissue septa were observed connecting the adjacent portal areas referred to as (Porto-portal fibrosis) resulting in formation of pseudo-lobules (thick white arrows), most hepatocytes appear with vacuolated cytoplasm and central dark nuclei (orange arrow), while some hepatocytes appear swollen with a single large cytoplasmic vacuole and peripheral nuclei (pink arrow). Areas of ruptured fused hepatocytes with indistinct cell boundaries (F) are also seen. PT; portal tract. CV; central vein, PV; portal vein, BD bile duct. [H & E scale bar (A, C, E) 200 μ m (B, D) 50 μ m, and (F) 100 μ m].

On the other hand, rats treated with SeNPs with or without diet modification (Groups IIIA & IIIB respectively) showed restored hepatic architecture like control groups, with some areas of hepatocyte affection, in the form of macro-steatosis. In addition, the group that continued HFD showed few focal areas of degenerated cells and minimal persistent cellular infiltration. Signs of hepatic regeneration were seen in both treatment groups such as apparent increase in the number of binucleated hepatocytes and increase in the cell plate thickness. (NAS scoring system subgroup III A scored: 3 while subgroup III B scored: 1. **(Figure 3 A, B, C & D)**

Meanwhile, liver sections of the spontaneous recovery group (group IV), (that received HFD for 8 weeks and continued with normal rat chow till the 10th week) showed distorted hepatic architecture with obliterated blood sinusoids between the severely disorganized cells. Central veins and sinusoids appeared congested with extravasated RBCs. Most hepatocytes

appear shrunken and hyper-eosinophilic, while a few cells depicted large single cytoplasmic vacuoles with peripheral flattened nuclei (NAS scoring system: 5. Pronounced cellular infiltration was seen around central veins, intercellular and peri-portal, with the appearance of porto-portal fibrous tissue septa (Fibrosis stage F3). **(Figure 3 E & F)**

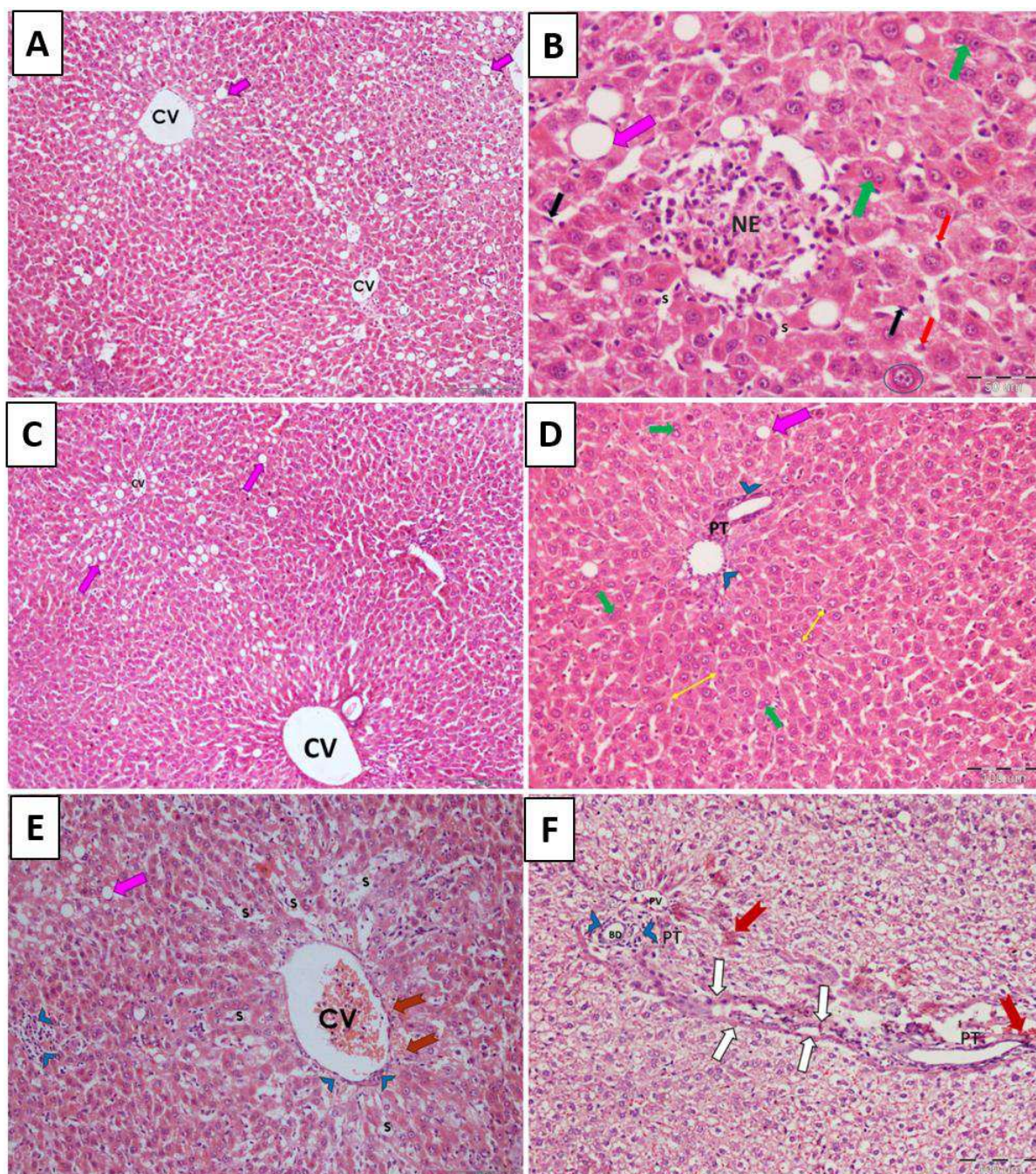


Fig. 3: Photomicrographs of both treatment (SeNPs with or without diet modification) and recovery groups: A & B treated with SeNPs and continued HFD till the 10th week, showing restored hepatic architecture in most areas around

the central vein while some areas show persistent cellular disarray. Most hepatocytes are arranged in cords radiating from the central veins (CV), separated by blood sinusoids (s) which are lined by endothelial cells (black arrow) and von Kupffer cells (red arrow). Hepatocytes appeared with eosinophilic granular cytoplasm, many of them appear binucleated (green arrow), while other cells depicted large nuclei containing numerous nucleoli (encircled). Small areas still show hepatocyte affection, in the form of macro-steatosis (pink arrow) and a few focal areas of degenerated cells with cellular infiltration (NE) were also seen. **C & D:** Photomicrograph of liver section from rats group (SeNPs+ diet modification), showing complete restoration of the hepatic architecture; hepatocytes are arranged in cords radiating from the central veins (CV) and separated by blood sinusoids appearing polyhedral in shape with granular eosinophilic cytoplasm, central vesicular nuclei, and prominent nucleoli. Some cells are binucleated (green arrow). Notice the increase in the cell plate thickness in some areas (double-headed yellow arrow). Only very few hepatocytes appear with eccentric nuclei and a single large vacuole (pink arrow). Minimal periportal (PT) cellular infiltration is also seen (blue arrowhead). **E & F:** Photomicrograph of liver section from rat group (spontaneous recovery) that received HFD for 8 weeks and continued with normal rat chow till the 10th week, showing loss of distorted hepatic architecture with obliterated blood sinusoids between the severely disorganized cells. Many central veins (CV) appear congested with extravasated RBCs (red arrow) and dilated congested blood sinusoids which reveal many von Kupffer cells (s). Extravasated RBCs (red arrow) are also seen around the portal tract and between the liver cells. Most hepatocytes appear shrunken and hyper-eosinophilic, while few cells depict large single cytoplasmic vacuole with peripheral flattened nuclei (pink arrow). Cellular infiltration is seen around the central vein, intercellular and peri portal (blue arrowhead). Note the Two adjacent portal tracts (PT) are joined together by eosinophilic fibers and flattened cells with flattened nuclei (thick white arrow). PT; portal tract. PV; branch of the portal vein. BD; branch of the bile duct. [H&E scale bar (A & C) 200 μ m (E, D&F) 100 μ m (B) 50 μ m].

3.3.2 Assessment of the degree of fibrosis using trichrome stain

Trichrome staining of control liver sections showed only scant connective tissue and collagen around portal tracts and central veins (**Figure 4 A**). In contrast, high-fat diet (HFD) groups exhibited progressive collagen deposition, ranging from moderate periportal fibrosis to extensive pericentral and bridging fibrosis with pseudo-lobulation and collagen accumulation between hepatocytes (**Figure 4 B & C**). Treatment with SeNPs markedly reduced collagen deposition, particularly around portal tracts and central veins. This effect was most pronounced in rats that received SeNPs with normal diet, where architecture closely approached the control pattern (**Figure 4E**), while rats that continued HFD alongside SeNPs showed reduced but still detectable periportal collagen (**Figure 4D**). The spontaneous recovery group showed partial improvement, with persistent moderate to extensive collagen deposition in some areas, indicating incomplete reversal of fibrosis (**Figure 4 F**).

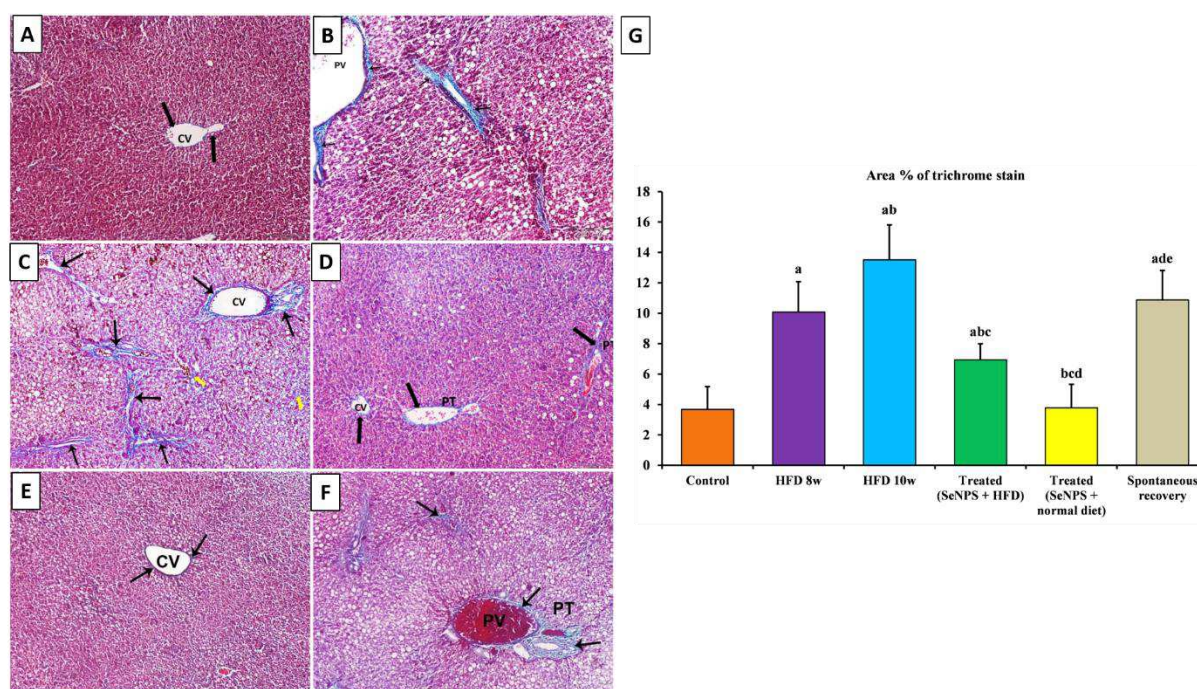


Fig. 4: Assessment of the degree of collagen deposition (fibrosis) using Gomori's trichrome stain: Gomori's trichrome staining of control liver sections showed only scant connective tissue and collagen around central vein (A). Moderate to severe fibrosis was observed in the HFD subgroups 8 weeks (B) and 10 weeks (C), while a marked reduction in collagen deposition was noted in the SeNPs-treated

subgroups (D)—especially in the SeNPs + normal diet group (E) comparable to the control groups, in contrast, persistent fibrosis was still evident in the spontaneous recovery group (F). These results were further supported by morphometric analysis, which measured the area percentage of trichrome-stained section (G). These findings confirm the antifibrotic effect of SeNPs.

3.3.3 Assessment of the degree of steatosis using ORO and osmium tetroxide stains

Oil Red O–stained liver sections from both control groups (subgroup I A & B) demonstrated hepatocytes containing only small red lipid droplets, indicating normal hepatic fat content (**Figure 5 A**). Hepatocyte nuclei appeared basophilic due to counterstaining. In subgroup IIA (HFD for 8 weeks), many hepatocytes exhibited numerous intracellular red lipid droplets of variable sizes, reflecting increased hepatic fat accumulation (Figure 5B). This finding was more pronounced in subgroup IIB (HFD for 10 weeks), in which the majority of hepatocytes were densely filled with lipid droplets, including large droplets indicative of marked steatosis (**Figure 5C**). In subgroup IIIA (SeNPs + HFD), liver sections showed heterogeneous lipid distribution, with some hepatocytes containing only few lipid droplets, while others remained occupied by numerous droplets of varying sizes (**Figure 5D**). Subgroup IIIB (SeNPs + diet modification) demonstrated hepatic lipid patterns comparable to control groups, with most hepatocytes showing minimal lipid droplets and only occasional cells containing small cytoplasmic droplets (**Figure 5E**). In group IV (spontaneous recovery), some hepatocytes exhibited reduced lipid accumulation; however, many cells still contained numerous cytoplasmic lipid droplets of variable sizes, indicating persistent hepatic fat deposition (**Figure 5F**). These results were further confirmed by osmium tetroxide staining of intracellular lipid (**Figure 6**).

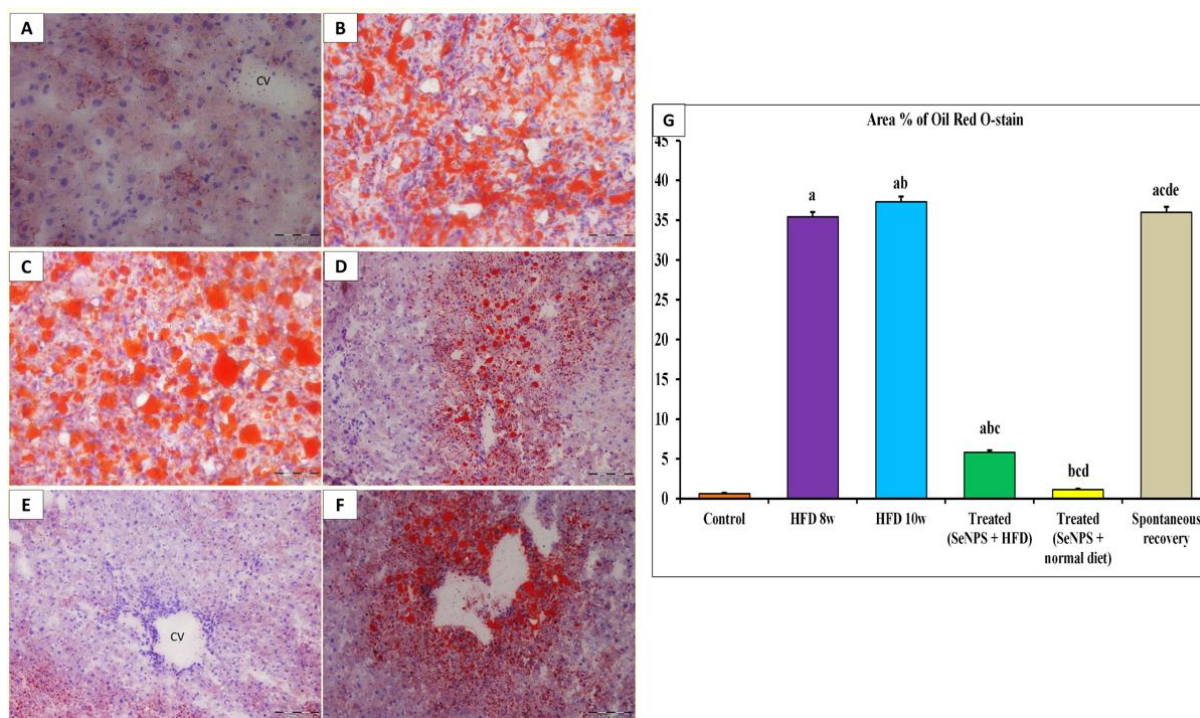


Fig. 5: Assessing the degree of steatosis using Oil Red O (ORO) stained frozen sections: Lipid deposits appeared red stained droplets in few hepatocytes in the control groups (A). In contrast, the HFD groups displayed the highest fat content (B, HFD for 8 weeks & C, HFD for 10 weeks), indicating marked steatosis. Treatment with SeNPs showed a marked reduction in lipid accumulation (D), while SeNPs + normal diet subgroup reached levels comparable to the control groups (E). However, persistent steatosis was still evident in the spontaneous recovery group (F). These findings were further confirmed by morphometric analysis, which measured the area percentage of ORO stained sections (G).

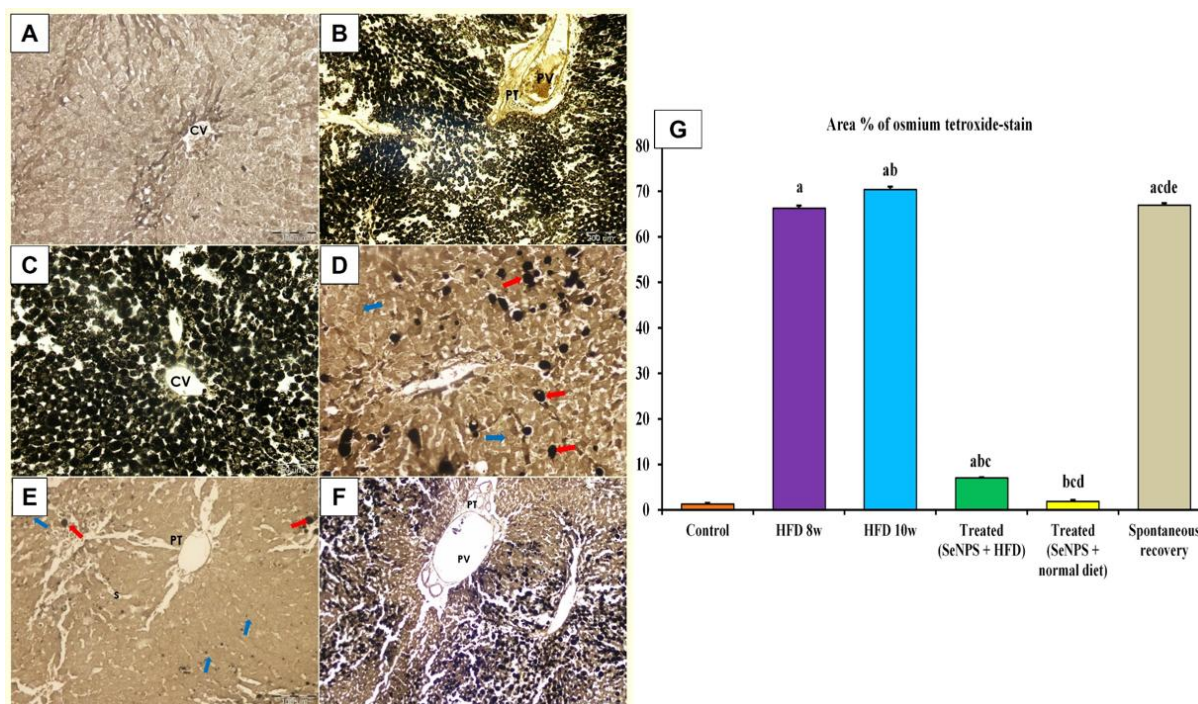


Fig. 6: Assessing the degree of steatosis using osmium tetroxide (OST) stained sections: where lipids appeared black. The highest fat content was observed in the HFD groups (B & C) 8 and 10 weeks respectively, indicating severe steatosis. In contrast, SeNPs-treated subgroups showed a marked reduction in fat accumulation (D), with the SeNPs + normal diet subgroup reaching levels comparable to control groups (E). However, persistent steatosis was still evident in the spontaneous recovery group (F). These results were further supported by morphometric analysis, which measured the area percentage of osmium tetroxide (OTO)-stained sections (G). CV; central vein, PT portal tract, PV; portal vein, blue arrows cells with normal fat content, red arrows with cells high fat content.

3.4 Electron microscopic study

Electron micrograph examination of rat **liver of both control groups** revealed normal hepatic architecture. Hepatocyte showed euchromatic nuclei with prominent nucleoli. Numerous mitochondria, profiles of rough and smooth endoplasmic reticulum and rosettes of glycogen granules were seen in the cytoplasm. Bile canaliculi appear bounded by desmosomes and enclosed between adjacent hepatocyte. The sinusoidal surface of the hepatocyte showed numerous microvilli. (**Figure 7 A&B**)

On the other hand, Electron micrograph of rat liver of high fat diet groups, revealed marked alterations of the hepatocyte ultrastructure. Hepatocytes with multiple lipid droplets some of which are fused were observed. Various mitochondrial anomalies such as electron-dense mitochondria, others are bizarre or ring shape was seen. Several hepatocytes appeared irregular and shrunken with eccentric small irregular nucleus and clumped peripheral heterochromatin surrounded by amalgamated organelles. The bile canaliculi between the hepatocytes appeared dilated and bounded by desmosomes. The presence of the thick collagen fibers was seen. **(Figure 7 C & D).**

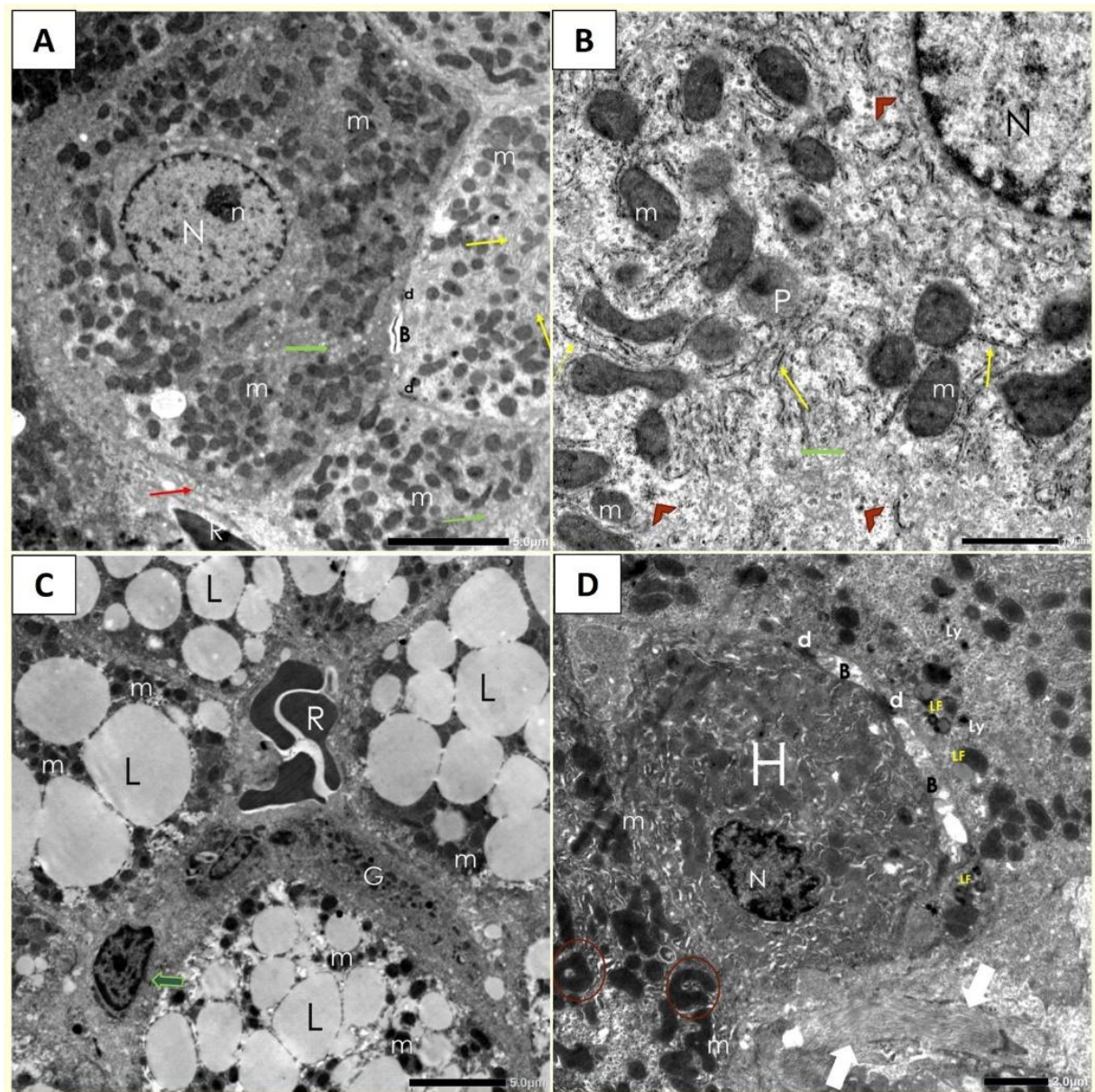


Fig. 7: Electron micrographs of rat liver from control and high- fat diet (HFD) groups:

A & B: Electron micrograph of rat liver subgroup IA (control 8w), revealing hepatocytes with numerous mitochondria (m), profiles of rough endoplasmic reticulum (rER) (yellow arrow) and smooth endoplasmic reticulum (sER) (green arrow). A regular euchromatic round nucleus (N) with a prominent nucleolus (n) is seen. A bile canaliculus (B), which is bounded by desmosomes (d) and enclosed between adjacent hepatocytes, is depicted. rosettes of glycogen granules (red arrowhead). The sinusoidal surface of the hepatocyte shows numerous microvilli (red arrow). R; RBC inside the sinusoids P; Peroxisome.

C: Electron micrograph of rat liver subgroup IIA (HFD 8w), showing hepatocytes containing electron-dense mitochondria (m) and multiple lipid droplets (L). Some of the lipid droplets are fused (F). A hepatocyte's nucleus (N) with eccentric nucleolus (n) is also seen. Notice the von Kupffer cell (K) is projecting in the narrowed sinusoid; deformed RBCs in the blood sinusoid.

D: Electron micrograph of rat liver subgroup IIB (HFD 10w), revealing an irregular shrunken shaped hepatocyte (H) with eccentric small irregular nucleus (N) and clumped peripheral heterochromatin surrounded by amalgamated organelles. In the neighboring cells, mitochondria (m) appear electron-dense. Some mitochondria are bizarre or ring shaped (red circle). The bile canaliculi (B) between the hepatocytes appear dilated and bounded by desmosomes (d). Notice the presence of the thick collagen fibers (white thick arrow). Ly; lysosomes. Lf; lipofuscin.

(Uranyl acetate/lead citrate stain, **A & C:** Mic. Mag. $\times 1500$, **B:** Mic. Mag. $\times 5000$, **D:** Mic. Mag. $\times 3000$)

Meanwhile, ultrastructural examination of both SeNPs treated groups (with or without diet modification) showed hepatocytes with normal ultrastructural appearance regular euchromatic nuclei and prominent nucleolus. The cytoplasm shows few small lipid droplets, with numerous partially preserved mitochondria, and profiles of rER. Hepatic stellate cells (HSC) with its characteristic cytoplasmic lipid droplets were seen in the space of Disse. The

bile canaliculi appeared with a normal diameter surrounded by intact desmosomes. In the group that continued HFD a few hepatocytes still exhibited high lipid content, and dilated partially degranulated rER was also seen (**Figure 8 A,B,C &D**).

In contrast, Electron micrograph of rat liver of spontaneous recovery group revealed persistent affection of most hepatocytes. The cytoplasm shows areas of rarefaction, multiple mitochondria with electron-dense matrix and multiple large lipid droplets some are fused together similar to those seen in HFD group. A few cells depicted nuclear vacuoles. (**Figure 8 E&F**)

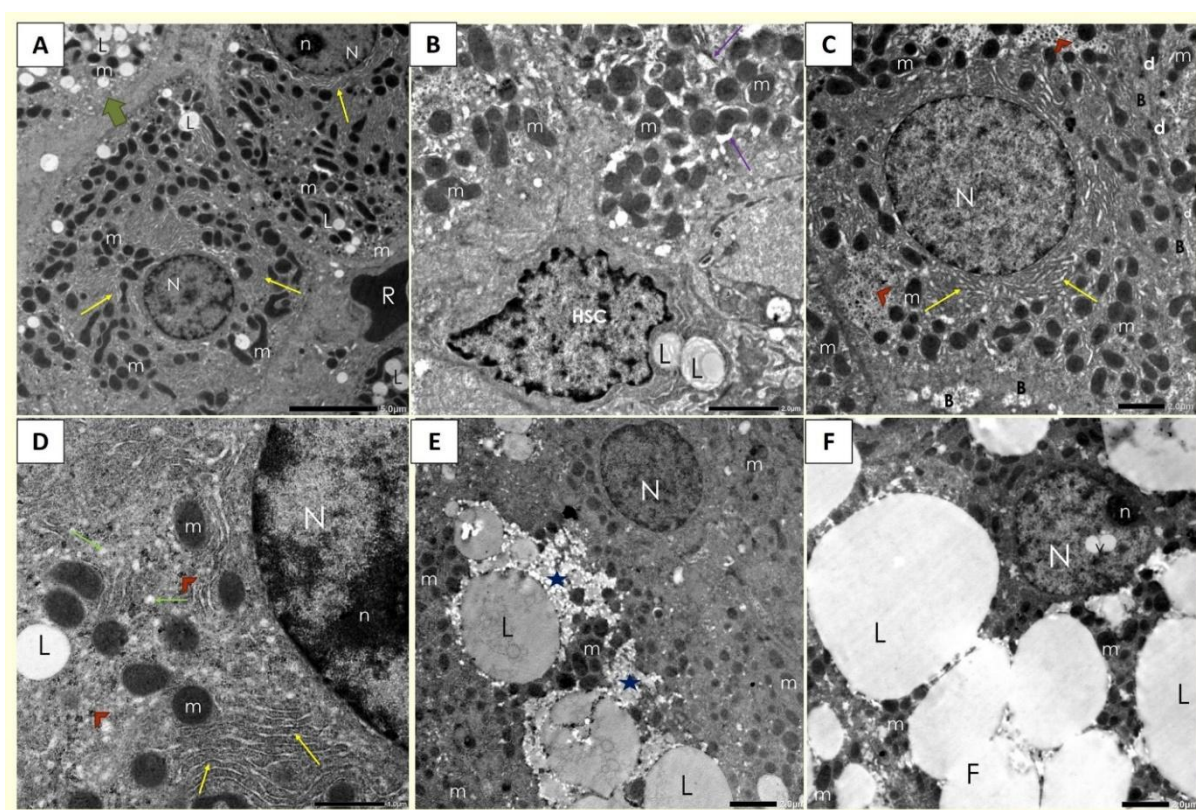


Fig. 8: Electron micrographs of rat liver treated groups and spontaneous recovery:

A: Electron micrograph of rat liver subgroup IIIA (SeNPs + HFD), showing hepatocytes with regular euchromatic nuclei (N) and prominent nucleolus (n). The cytoplasm shows few small lipid droplets (L), with numerous partially preserved mitochondria (m), and profiles of rER (yellow arrow). Notice that one hepatocyte still contains high lipid content (thick green arrow).

B: Electron micrograph of rat liver subgroup IIIA (SeNPs + HFD), a part of two adjacent hepatocytes that exhibit partial improvement showing numerous mitochondria (m),

dilated partially degranulated rER (purple arrow). Notice the hepatic stellate cell (HSC) with its characteristic cytoplasmic lipid droplets (L).

C & D: Electron micrograph of rat liver subgroup IIIB (SeNPs + normal diet), revealing recovery of hepatocyte with a regular spherical euchromatic nucleus (N). The cytoplasm shows numerous mitochondria (m), parallel cisternae of rER (yellow arrow), smooth ER (green arrow) and rosettes of glycogen (red arrowhead) similar to those seen in the control group. Notice the presence of the bile canaliculi (B) surrounded by desmosomes (d) between the hepatocyte and its neighboring cells. Lipid droplet (L).

E & F: Electron micrograph of rat liver of group IV (spontaneous recovery): revealing a hepatocyte with a spherical nucleus (N). The cytoplasm shows areas of rarefaction (blue star), multiple mitochondria with electron-dense matrix (m), and multiple large lipid droplets (L) some are fused together (F) similar to those seen in HFD group. Notice the nuclear vacuoles (v) seen in figure F.

(Uranyl acetate/lead citrate stain A: Mic. Mag. $\times 1500$, B, C, E & F: Mic. Mag. $\times 3000$, D: Mic. Mag. $\times 5000$)

3.5 Morphometric and quantitative results

3.5.1 Morphometric analysis for collagen fibers deposition in Masson's trichrome stained

In the present study, all the HFD subgroups showed a significant increase in mean area percentage of collagen compared to the control groups ($p \leq 0.001$), with the highest level observed in the HFD 10 w subgroup (mean=13.52). Conversely, a statistically significant decrease in collagen area percentage was observed in all the treatment subgroups compared to the HFD subgroups ($p \leq 0.001$), with levels showing no statistically significant difference from the control groups in the SeNPs + normal diet treatment subgroup ($p=1.000$). In the spontaneous recovery group, collagen area percentage remained significantly elevated compared to the control groups ($p \leq 0.001$), with no significant difference from any of the HFD subgroups; HFD 8 w and HFD 10 w ($p = 0.968$ and 0.132), respectively (**Figure 4G**).

3.5.2 Morphometric analysis of area percentage (%) of steatosis in Oil-Red-O (ORO) stained liver sections:

In the present study, there is a significant increase in the mean area percentage of Oil Red O (ORO) staining was observed in all high-fat diet (HFD) subgroups compared to the control group ($p \leq 0.001$). The highest value was recorded in the HFD 10w subgroup (mean = 37.32), which showed a significantly greater stained area percentage than the HFD 8w subgroup ($p \leq 0.001$). In contrast, all the treatment subgroups exhibited a significant reduction in ORO-stained area percentage compared to the HFD group ($p \leq 0.001$). Notably, the (SeNPs + normal diet) treatment subgroup showed no significant difference from the control groups ($p = 0.402$). Additionally, the spontaneous recovery group displayed a persistently elevated stained area percentage compared to the control groups ($p \leq 0.001$), with no significant difference observed between this group and the HFD 8 w subgroup ($p = 0.310$) (**Figure 5 G**).

3.5.3 Morphometric analysis for area % of steatosis in osmium tetroxide-stained liver

In this study, a significant increase in the mean area percentage of osmium tetroxide stained sections were observed in all high-fat diet (HFD) subgroups compared to the control groups ($p \leq 0.001$), with the highest value recorded in the HFD 10 w subgroup (mean=70.39). Conversely, all the treatment subgroups exhibited a significant reduction in staining compared to the HFD groups ($p \leq 0.001$), with levels showing no statistically significant difference from the control groups in the SeNPs + normal diet subgroup ($p=0.169$). The spontaneous recovery group maintained a significantly elevated staining area compared to the control groups ($p \leq 0.001$), with no significant difference from the HFD 8 w subgroup ($p=0.072$) (**Figure 6 G**).

3.6 Biochemical results

3.6.1 Liver Enzymes: SGOT and SGPT

In the current study, there was no statistically significant difference in the mean levels of SGOT and SGPT among the different control subgroups ($p \leq 0.05$). These enzyme levels were significantly elevated in all HFD subgroups and the spontaneous recovery group compared to the control group ($p \leq 0.001$). Meanwhile, SGOT and SGPT levels were significantly reduced

in both treatment subgroups compared to the HFD group ($p \leq 0.001$) and showed no significant difference from the control group ($p \leq 0.05$) group, and showed no significant difference from either HFD group, indicating persistent hepatic injury in the absence of targeted treatment **(Figure 9 A & B)**.

3.6.3 Lipid Profile: Total Cholesterol, Triglycerides, LDL, and HDL

No statistically significant differences were observed in the mean levels of total cholesterol (TC), triglycerides (TGs), low-density lipoprotein (LDL), or high-density lipoprotein (HDL) among the various control subgroups ($p \leq 0.05$). All high-fat diet (HFD) subgroups exhibited significant dyslipidemia compared to the control group: TC ($p \leq 0.001$), TGs ($p \leq 0.001$), and LDL ($p \leq 0.001$) were significantly elevated, while HDL was significantly reduced ($p \leq 0.001$). A duration-dependent effect was observed, with the HFD 10 w subgroup showing significantly higher TC ($p = 0.035$), TGs ($p = 0.047$), and LDL ($p = 0.023$), and lower HDL ($p = 0.022$) compared to the HFD 8 w subgroup.

Treatment with selenium nanoparticles (SeNPs) significantly improved all lipid parameters. Both the SeNPs + HFD and SeNPs + normal diet subgroups demonstrated significant reductions in TC, TGs, and LDL compared to the HFD subgroups ($p \leq 0.001$ for all). The SeNPs + normal diet subgroup further showed a significant decrease in TC ($p = 0.003$), TGs ($p = 0.002$), and LDL ($p = 0.011$), and but no significant increase in HDL ($p = 0.112$) compared to the SeNPs + HFD subgroup. Notably, the SeNPs + normal diet subgroup restored TC ($p = 0.937$), TGs ($p = 0.992$), LDL ($p = 0.878$), and HDL ($p = 0.999$) to levels statistically indistinguishable from the control group.

In contrast, the spontaneous recovery group exhibited a lipid profile that remained significantly disrupted: TC ($p \leq 0.001$), TGs ($p \leq 0.001$), and LDL ($p \leq 0.001$) were elevated compared to controls, while HDL was reduced ($p \leq 0.001$). This group showed no significant improvement relative to the HFD groups, indicating that spontaneous dietary reversion alone was insufficient to normalize HFD-induced dyslipidemia. (Figure 9 C, D, E & F)

3.6.4 Glycemic Parameters: Fasting Plasma Glucose (F P G), Fasting insulin and HOMA-IR

No significant differences were observed in mean fasting plasma glucose (FPG), fasting insulin, or HOMA-IR levels among the control subgroups. Both high-fat diet (HFD) groups, 8

weeks (HFD8w) and 10 weeks (HFD10w) exhibited significantly elevated levels of all three parameters compared to the control group ($p \leq 0.001$).

There was no statistically significant difference in FPG between the two HFD durations, and similarly, fasting insulin levels did not differ significantly between HFD8w and HFD10w ($p \leq 0.05$). However, HOMA-IR was significantly higher in HFD 10w compared to HFD8w ($p = 0.005$).

Treatment with selenium nanoparticles (SeNPs) significantly ameliorated these disturbances: the SeNPs + HFD group showed a significant reduction in FPG ($p = 0.023$, $p < 0.001$), fasting insulin ($p = 0.001$, $p \leq 0.001$), and HOMA-IR ($p = 0.001$, $p \leq 0.001$) compared to HFD groups at 8 weeks and 10 weeks respectively.

Notably, the SeNPs + normal diet group exhibited no significant difference in FPG ($p = 0.955$), fasting insulin ($p = 0.999$), or HOMA-IR ($p = 1.000$) when compared to the control group, indicating a return to baseline glycemic homeostasis.

In contrast, the spontaneous recovery group demonstrated significantly increased FPG ($p \leq 0.001$), fasting insulin ($p \leq 0.001$), and HOMA-IR ($p \leq 0.001$) relative to controls, with values remaining comparable to those of the HFD groups with FPG ($p = 0.999$, $p = 0.021$), fasting insulin ($p = 0.504$, $p = 0.890$) and HOMA-IR ($p = 0.734$, $p = 0.123$) compared to 8 weeks and 10 weeks HFD respectively, reflecting a lack of metabolic improvement without intervention. **Figure 9 G, H & I.**

3.6.5 Oxidative Stress Markers: Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Total Antioxidant Capacity (TAC)

No statistically significant differences were observed in the mean tissue levels of malondialdehyde (MDA), superoxide dismutase (SOD), or total antioxidant capacity (TAC) among the various control subgroups ($p \leq 0.05$). All high-fat diet (HFD) subgroups exhibited a

pronounced state of oxidative stress, characterized by significantly elevated MDA levels ($p \leq 0.001$) and significantly reduced SOD ($p \leq 0.001$) and TAC ($p \leq 0.001$) compared to the control group. No significant differences in these markers were observed between the HFD8w and HFD10w subgroups (MDA: $p = 0.176$; SOD: $p = 0.358$; TAC: $p = 0.088$).

Treatment with selenium nanoparticles (SeNPs) significantly mitigated oxidative damage. Both the SeNPs + HFD and SeNPs + normal diet subgroups showed a significant reduction in MDA ($p \leq 0.001$) and a significant increase in SOD ($p \leq 0.001$) and TAC ($p \leq 0.001$) compared to both HFD subgroups. The SeNPs + normal diet subgroup was particularly effective, restoring SOD ($p < 0.001$), and TAC ($p = 0.040$) to levels that significantly improved towards control values. Furthermore, the SeNPs + normal diet subgroup showed a statistically significant improvement in MDA ($p = 0.039$), SOD ($p = 0.016$), and TAC ($p < 0.001$) compared to the SeNPs + HFD subgroup, indicating a superior antioxidant effect when combined with a normal diet.

In contrast, the spontaneous recovery group demonstrated no amelioration of oxidative stress. This group maintained significantly elevated MDA ($p \leq 0.001$) and reduced SOD ($p \leq 0.001$) and TAC ($p \leq 0.001$) compared to the control group, with levels showing no significant difference from any of the HFD subgroups. This indicates that cessation of the HFD without SeNPs intervention was insufficient to reverse diet-induced oxidative damage (**Figure 9 J, K & L**).

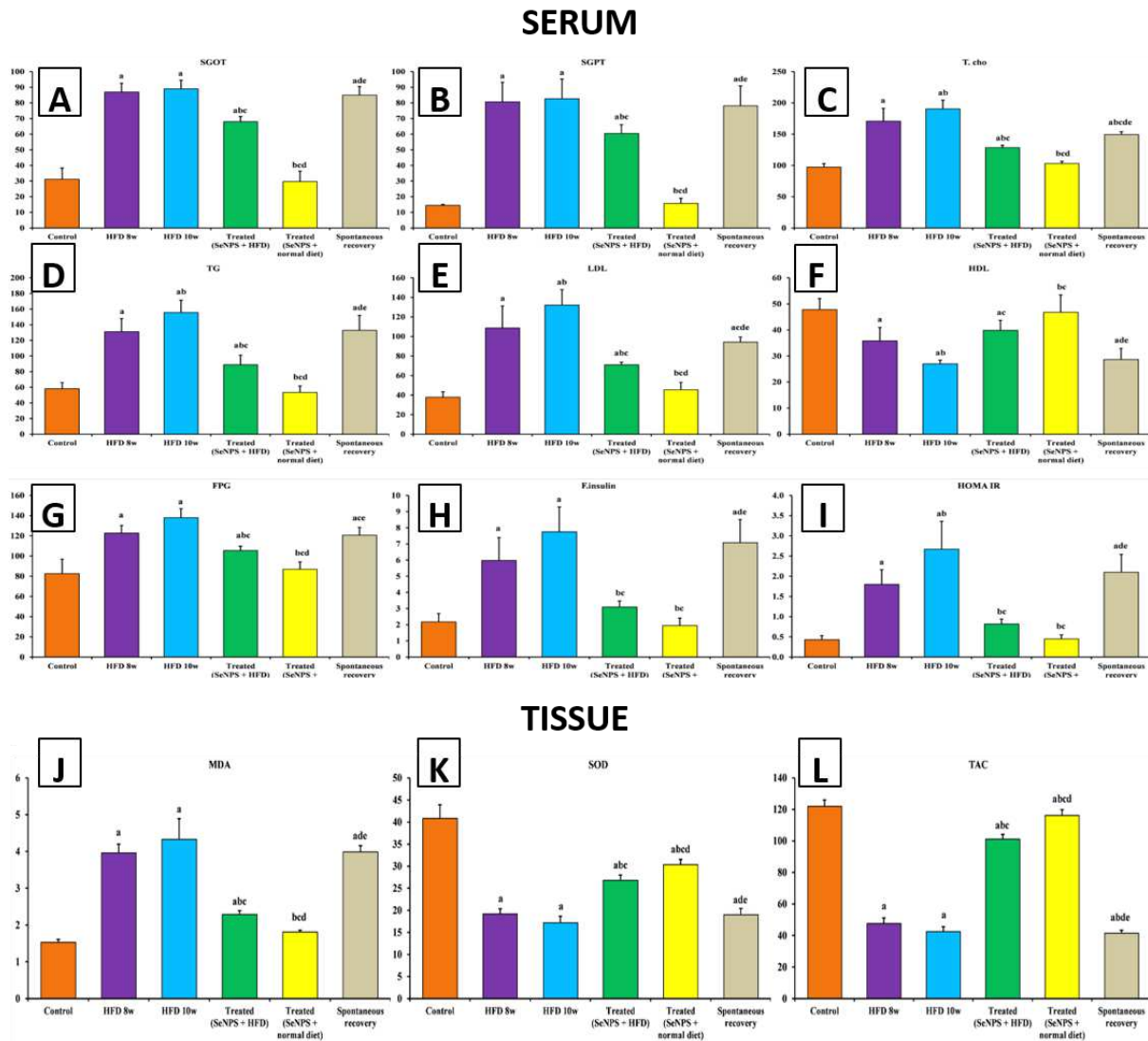


Fig. 9: Comparison between the different studied groups according to serum A) SGOT, B) SGBT, C) Total cholesterol, D) Triglyceride, E) LDL, F) HDL, G) Fasting blood glucose H) fasting insulin, I) HOMA IR, and tissue J) Tissue Malondialdehyde (MDA) levels, K) Tissue Superoxide dismutase (SOD) levels, L) Tissue Total antioxidant capacity (TAC) levels. Statistically significant at $p \leq 0.05$. a: Significant with Control b: Significant with HFD (8w) c: Significant with HFD (10w) d: Significant with SeNPS + HFD e: Significant with SeNPS + normal diet.

3.6.6 Hepatic Fibrosis markers: Smooth muscle actin (SMA) and transforming growth factor beta (TGF- β)

SMA expression was significantly elevated in the HFD 8w (2.78 ± 1.02) and HFD 10w (3.36 ± 1.08) groups compared to the control group (1.02 ± 0.19 ; $F=12.918$, $p<0.001$). A time-dependent increase was observed, with the HFD 10w group showing the highest SMA expression, although the difference between the two HFD durations did not reach statistical significance ($p=0.923$).

Treatment with SeNPs significantly reduced SMA expression in both the SeNPs + HFD (1.34 ± 0.14) and SeNPs + normal diet (1.08 ± 0.17) subgroups compared to the HFD 10w group ($p=0.022$ and $p=0.012$, respectively). Moreover, the SeNPs + normal diet subgroup showed no significant difference from the control group ($p=0.991$), reflecting complete normalization of fibrotic markers when combining SeNPs with dietary modification.

On the other hand, spontaneous recovery group maintained significantly elevated SMA (2.23 ± 0.50) compared to controls ($p=0.009$) and remained significantly higher than the SeNPs + normal diet group ($p=0.042$), indicating incomplete resolution of fibrosis with dietary modification alone.

Similarly, TGF- β expression showed a significant difference across groups. While both HFD groups exhibited elevated TGF- β expression, the differences did not reach statistical significance compared to controls. However, the SeNPs + HFD group demonstrated significantly lower TGF- β compared to the HFD 10w group ($p=0.002$), and the SeNPs + normal diet group showed significantly lower TGF- β compared to both HFD 8w ($p=0.001$) and HFD 10w ($p=0.002$). These findings further confirm the anti-fibrotic effects of SeNPs, especially when combined with dietary modifications. **(Figure 10)**

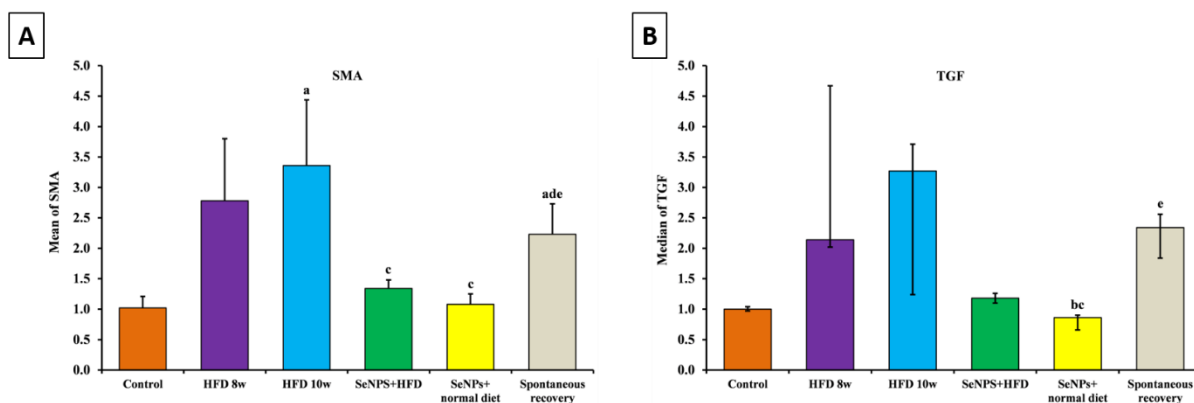


Fig. 10: Comparison between the different studied groups according to SMA and TGF- B respectively. Statistically significant at $p \leq 0.05$. a: Significant with Control b: Significant with HFD (8w) c: Significant with HFD (10w) d: Significant with SeNPS + HFD e: Significant with SeNPs + normal diet.

4. DISCUSSION

Despite non-alcoholic fatty liver disease (NAFLD) widespread impact, effective pharmacological treatments remain limited, necessitating the exploration of novel therapeutic interventions. Dietary modifications alone may not be sufficient to fully reverse NAFLD-associated liver damage.^[20]

The use of Nano-selenium has become a point of interest in various disease treatment due to its higher biocompatibility and lower toxicity than other Selenium compounds.⁶ Since selenium plays a vital role in regulating hepatic lipid metabolism, oxidative stress and inflammation therefore, selenium nanoparticles (SeNPs) are assumed to have considerable promise in the treatment of NAFLD due to their distinctive properties.^[8]

The current study aimed to evaluate the potential therapeutic effect of SeNPs with or without dietary modification on the course of hepatic pathology in high fat diet induced NAFLD.

The in-house formulated high-fat diet (HFD) (58% calories as fat) model used in the current study is one of the most commonly used methods for the induction of NAFLD.^[19] In

addition, this model is simple, reproducible, reliable, and affordable, hence ideal for the development of new therapeutic targets.^[21] Adult male albino rats were chosen for this study since they are more susceptible than mice to develop HFD induced NAFLD, with a shorter time needed to induce the histological manifestations.^[22]

In the current study, Animals that received SeNPs (subgroup IB) showed the same normal histological structure as the other control subgroups, indicating the safety of the used dose, size, and method of administration of SeNPs used in the present work.

In this study, the light microscopic examination of the livers of the animals fed with HFD for 8 weeks (subgroup IIA) and 10 weeks (subgroup IIB) revealed loss of normal hepatic architecture with a mild to evident degree of fibrosis, respectively. Hepatocytes displayed variable degrees of steatosis and degeneration involving widespread segments of several hepatic lobules in subgroup IIA, while it was more marked in subgroup IIB, involving most of the hepatic lobules. These findings were aligned with previous studies that reported HFD administration for a longer period (10 weeks) or more resulted in severe damage to the hepatocytes and their organelles.^[23, 24]

Hepatocellular steatosis is a major characteristic of NAFLD, which occurs when over 5% of liver cells contain fat in the form of triglycerides (TGs) generally starts in zone 3 of the liver and can affect the entire acinus in severe cases.^[23] Vacuolation of hepatocytes seen in our study in group II (HFD) was described as micro-vesicular and macro-vesicular steatosis. Micro-vesicular steatosis refers to the accumulation of tiny fat droplets within the liver cells that do not displace the nuclei, while macro-vesicular steatosis refers to hepatocytes with a large single fat droplet or smaller well-defined droplets displacing the nuclei to the cell periphery.^[24]

In the present study, the steatosis was confirmed by Oil-Red-O (ORO) staining, as it is the “gold standard” histochemical fat-soluble dye for specifically identifying neutral lipids, especially TGs.^[25] These findings were also confirmed by osmium tetroxide staining. Both

stains were employed to detect intracellular hepatic fat, consistent with previous studies.^[26, 27] In this study, steatosis was staged according to the most widely used staging system, NAFLD Activity Score (NAS), by two blinded observers.^[13] Accordingly, subgroup IIA (HFD 8 w) was scored as stage 2 steatosis, which is defined as involvement of 33 to 66 percent of the liver sections. While the (HFD 10w) subgroup was scored as stage 3 steatosis, which involves more than 66 percent. These results were further confirmed by morphometric analysis of ORO and Osmium tetroxide, which revealed a significant increase in area percentage of affection of both subgroups when compared to the control groups, with more significant affection in the (HFD 10w) subgroup.

The hepatocellular injury in NAFLD is explained by two interrelated mechanisms: direct cellular lipotoxicity and indirect damage through lipid peroxidation. The direct mechanism involves the accumulation of excess intracellular fatty acids, which promotes the generation of reactive oxygen species (ROS) that damage cellular components such as mitochondria, rER, and other organelles. In parallel, the indirect mechanism results from the cytotoxic effects of abnormal lipid peroxidation products, which further exacerbate cellular injury. Both processes converge to create a state of oxidative stress, widely recognized in experimental studies as the central driver of liver cell damage in NAFLD.^[28, 29] All of which will lead to hepatocellular degenerative changes, including ballooning degeneration, which is the hallmark of NASH and has been associated with more aggressive disease and a higher incidence of hepatic cirrhosis.^[28]

In the current work, ballooning degeneration of hepatocytes was seen in experimental animals of group II (HFD) Subgroup IIA (NAS score 1), while subgroup IIB showed prominent ballooning (NAS score 2). Hepatocytic ballooning was previously attributed to mitochondrial damage as a result of lipotoxicity, which leads to reduction of the intracellular ATP, hence dysfunction of the membranal Na^+/K^+ pump, causing an increase of the intracellular Na^+ and water retention.

Some of the ballooned hepatocytes of group II (HFD) showed Mallory-Denk bodies (MDBs), which are defined as intracellular deposition of misfolded intermediate filaments (mainly keratin 8 & 18).^[30] Both hepatocyte ballooning and MDBs are essential features of an ongoing inflammation.^[31]

In the current study, some focal areas in the liver sections of animals of subgroup IIB revealed the presence of necrotic hyper-eosinophilic hepatocytes and areas of fused hepatocytes with indistinct cell boundaries, which appeared predominantly in the centrilobular regions (zone 3) of the liver. This finding may be due to the region's relatively low oxygen supply and high cytochrome P450 activity, making hepatocytes more susceptible to oxidative stress and toxic metabolite accumulation.^[32, 33] The increased eosinophilia is attributed to the loss of normal basophilia caused by cytoplasmic RNA, and to the increased binding of the eosin to the intracytoplasmic denatured proteins.^[34, 35] Unlike apoptosis, which is a regulated form of cell death, necrosis in NAFLD reflects a more severe and uncontrolled injury, indicating progression toward non-alcoholic steatohepatitis (NASH) and potentially cirrhosis.^[33]

Signs of inflammation, as mononuclear cellular infiltration, were also seen in both of the HFD subgroups around the central veins, portal tracts, and between the hepatocytes: subgroup IIA (NAS score 2) and subgroup IIB (NAS score 3). This is considered one of the diagnostic features of steatohepatitis.^[36]

Fibrosis was seen in group II (HFD) but was more pronounced in subgroup IIB (HFD 10w), with formation of pseudo lobules. (Stage 3 fibrosis F3). Collagen was also increased surrounding the central veins. These findings were verified using trichrome stain and morphometric study, similar findings reported in previous studies on fatty liver.^[37, 38]

The ultrastructure examination by TEM of liver sections from both the HFD subgroups (HFD8w & HFD10w) revealed significant mitochondrial alterations, characterized by an electron-dense matrix and loss of cristae. The mitochondrial abnormalities observed in this study

are consistent with previous findings and have been attributed to impaired intramitochondrial protein synthesis, dysfunction of the respiratory chain, and elevated cytosolic calcium levels induced by oxidative stress.^[39, 40] The presence of enlarged mitochondria in some cells is thought to represent an adaptive response to oxidative stress or a consequence of suppressed mitochondrial division due to reduced protein synthesis.^[39-41] Additionally, some mitochondria appeared abnormally shaped, including ring-like forms. These structural abnormalities have been previously associated with increased severity of NAFLD and may underlie the elevated mitochondrial reactive oxygen species (ROS) production, as well as the enhanced lobular inflammation and hepatocyte ballooning seen in NASH.^[32]

Nuclear degenerative changes were seen in these group electron-dense nuclei with clumped heterochromatin. Some nucleoli appeared dense and eccentric in position. Margination of nucleoli to the periphery of the nucleus was explained as a means to promote nuclear-cytoplasmic exchange in the early stages of cell injury. As the injury continues, it becomes ineffective, and the nucleoli may finally degenerate.^[42, 43] A subset of nuclei demonstrated intranuclear vacuoles, most likely corresponding to lipid droplets. Intranuclear lipid droplets have been reported in various liver diseases, including nonalcoholic fatty liver disease (NAFLD); however, their functional significance remains unclear. Recent evidence suggests that endoplasmic reticulum (ER) stress suppresses very-low-density lipoprotein (VLDL) secretion, leading to its accumulation within the ER lumen. Subsequent translocation into the lumen of the nucleoplasmic reticulum may contribute to an increased number of nuclear lipid droplets.^[44]

Dilatation and proliferation of the bile ducts seen in both LM and ultrathin sections of group II (HFD), were previously described as a part of the ductular reaction (DR) to hepatic injury in various hepatic NAFLD models.^[37] Toxic injury to the liver frequently results in elevated bile secretion as a compensatory mechanism to clear harmful metabolic byproducts,

which in turn stimulates the growth and extension of bile ducts to enhance the efficiency of bile excretion.^[45] This may be as a result of either the proliferation of existing non-damaged ductules or liver cell differentiation into biliary-type cells.^[46] In addition to bile duct proliferation, DR involves infiltration of immune cells into the portal areas and the activation of hepatic progenitor cells (HPCs) with the formation of a ‘niche’ around them, including macrophages, myofibroblasts, and extracellular matrix components, all of which are closely linked to the development of liver fibrosis.^[47, 48] bile ductal cells may also activate latent transforming growth factor- β (TGF- β), which induces collagen production and deposition by hepatic stellate cells, further aggravating liver fibrosis.^[49]

The blood sinusoids appeared congested with blood. Extravasated RBCs and macrophages with pseudopodia engulfing them were also seen. These indicate a certain degree of vascular damage or increased permeability occurring in the microvasculature of the liver, which caused the blood vessels, particularly the liver sinusoids, to become leaky via cytokines (like TNF- α and IL-6), released from activated Kupffer cells which disrupt the structure and function of endothelial cells, allowing RBCs to escape into the liver tissue.^[4, 28]

In the NAFLD setting, it was postulated that metabolic stress, lipotoxicity, and sustained inflammation contribute to an altered hepatic niche, a specialized microenvironment within the liver that includes hepatocytes, hepatic progenitor cells (HPCs) and non-parenchymal cells (like Kupffer cells, hepatic stellate cells, sinusoidal endothelial cells), extracellular matrix, and signaling molecules.^[50]

EM examination of this group revealed the presence of cells in the perisinusoidal and periportal spaces. which were characterized by being oval shaped with a large nucleus and densely packed, highly clumped heterochromatin, particularly concentrated beneath the nuclear envelope. Their cytoplasm was relatively sparse, leading to a high nucleus-to-cytoplasm ratio. It was previously reported that these cells represent hepatic progenitor cells (HPCs), also known

as oval cells.^[51, 52] The interplay between HPCs and the inflammatory microenvironment dictates the outcome of hepatic injury. In severe liver damage, where hepatocyte regeneration is compromised, HPCs differentiate into hepatocytes and biliary cells to restore liver mass and function. Conversely, during chronic liver inflammation persistent HPCs proliferation has been strongly linked to increased disease severity and progressive fibrosis driven by their differentiation and enhanced extracellular matrix deposition through fibrogenic mechanisms resembling epithelial-to-mesenchymal transition (EMT).^[2, 22, 53] Moreover, prolonged activation of HPCs has also been implicated in an elevated risk of hepatocellular carcinoma (HCC).^[53]

Biochemical results in all the HFD subgroups went hand in hand with the previously mentioned histological results. They showed significantly elevated serum AST and ALT levels in comparison to the control groups. Which reflects liver cell injury caused by fat accumulation, oxidative stress, mitochondrial dysfunction, and inflammation. Other researchers also reported the same results of ALT & AST levels after HFD administration.^[38, 54]

Also, there was an increase in fasting glucose, insulin, and HOMA-IR in the NAFLD rat for 8 and 10 weeks compared to the control and treated groups, this finding is attributed mainly to diet-induced insulin resistance. Rats on a HFD showed high lipid buildup in the liver and fat tissues, disrupting insulin signaling. This made insulin less effective, causing higher blood glucose levels. The pancreas tried to keep glucose levels stable by producing more insulin, leading to hyperinsulinemia and higher HOMA-IR. Insulin resistance in the liver also continued glucose production. Additionally, fat tissue dysfunction raises free fatty acids, worsening lipid buildup, and metabolic stress. The chronic inflammation from a HFD further disrupts insulin signaling, creating conditions similar to human NAFLD. Similar findings were reported by other researchers who studied the association between NAFLD and Insulin resistance.^[55, 56]

The lipid profile results in the present study showed that feeding rats a high-fat diet significantly raised serum levels of TC, LDL, and TGs in comparison to the control and treated groups. This was again mainly due to the insulin resistance, which disrupts lipid metabolism. This imbalance reflects metabolic disturbances related to NAFLD, alongside a significant drop in HDL levels compared to the control groups. This may be due to the decrease in the enzyme involved in the transesterification of cholesterol and the maturation of HDL, causing the flux of cholesterol from cell membranes into HDL. Similar findings were reported by other authors, showed abnormal lipid profiles after the HFD administration.^[10, 22, 26]

In addition, the present work revealed a clear rise in tissue MDA levels, along with a noticeable drop in both SOD activity and TAC, confirming that oxidative stress plays a significant role in the liver changes seen in NAFLD-induced rats. The buildup of fat within liver cells likely triggered an overproduction of ROS, placing stress on the liver's natural antioxidant systems. As a key antioxidant enzyme, SOD seems to be either overwhelmed or depleted in this process. Similarly, the reduced TAC points to an overall exhaustion of the body's antioxidant defenses, both enzymatic and non-enzymatic. This imbalance between increased ROS and weakened antioxidant protection results in oxidative damage to lipids, as reflected by higher MDA levels. This lipid peroxidation not only damages cells but may also accelerate the progression of simple fatty liver to more severe liver disease. These findings are consistent with previous studies identifying oxidative stress as a key factor in development and progression of NAFLD.^[22, 57, 58]

In the current work, SeNPs were assessed for the potential to ameliorate hepatic injury induced by HFD. SeNPs were administered at a dosage of 2 mg/kg through oral gavage, as several studies have reported their potential therapeutic, hepatoprotective, and metabolic regulatory effects in this dose.^[11, 59, 60] The administration of SeNPs by oral gavage closely mimics the natural route of selenium intake in humans and allows interaction with the gut microbiota of the gut-liver axis as

a key pathway in NAFLD pathogenesis. In a very recent study, oral SeNPs have been shown to modulate gut microbiota, improve intestinal barrier integrity, and reduce endotoxin translocation, thereby attenuating hepatic inflammation and steatosis.^[61] Moreover, after absorption, SeNPs are delivered via the portal circulation directly to the liver, enhancing targeted hepatic effects.^[22] In the present study, SeNPs was administered for 2 weeks. This duration was consistent with previous research in preclinical models.^[12] Our findings suggest that even short-term treatment with SeNPs can elicit rapid biological responses, making them a promising candidate for early-phase intervention in NAFLD.

Examination of the LM sections of subgroup IIIA (SeNPs + HFD) showed that SeNPs attenuated the hepatic steatosis and decreased the severity of fibrosis and inflammation with partial restoration of the normal liver architecture, even when HFD administration continued. Residual focal areas of steatosis were still seen. In contrast, LM examination of the liver in subgroup IIIB (SeNPs + normal diet) showed marked regression of the inflammatory degenerative effects induced by HFD. The liver restored nearly its normal architecture and appeared more or less similar to the control groups. Most of the hepatocytes were polyhedral in shape with acidophilic granular cytoplasm and central vesicular nuclei. No hepatocyte ballooning was seen in both subgroups (NAS score 0). In addition, signs of liver regeneration were observed in group IIIB in the form of increased thickness of the cell plates, a high number of binucleated cells, and large nuclei containing numerous nucleoli.

This improvement was attributed to the hepatoprotective effects of selenium nanoparticles (SeNPs), which have been previously reported to exhibit potent antioxidant activity. SeNPs effectively neutralize reactive oxygen species (ROS) and enhance the activity of key antioxidant enzymes, including glutathione peroxidase (GPx) and superoxide dismutase (SOD).^[6, 62] These actions collectively mitigate oxidative stress, a major contributor to liver injury and the progression of nonalcoholic fatty liver disease (NAFLD).^[63] Consistent with

these findings, other studies have demonstrated the antioxidant effects of SeNPs across various disease models.^[64-67]

Reduction of hepatic steatosis was seen in all the treated subgroups, as only very few hepatocytes appeared to still contain the single large cytoplasmic vacuole and the peripheral nucleus. These findings were confirmed by ORO and osmium tetroxide stains. The results were evaluated by a morphometric study that showed a significant reduction in liver steatosis in the treated group compared to the HFD group. Also, it was more pronounced in subgroup IIIB (NAS score 0), when compared to subgroup IIIA (NAS score 1).

It has been reported that SeNPs can modulate lipid metabolism by enhancing insulin sensitivity and suppressing de novo lipogenesis in liver cells.^[61] They also aid in regulating the expression of key enzymes and genes involved in fatty acid synthesis and oxidation, including upregulation of lipid oxidative genes like peroxisome proliferator-activated receptors (PPARs), particularly PPAR- α , which promotes β -oxidation of fatty acids in the liver and reduces hepatic lipid accumulation, in addition to downregulation of lipogenic genes that promote hepatic de novo lipogenesis.^[68] The net effect of these mechanisms contributes to the reduction in hepatic lipid content observed in the SeNPs-treated group.

In the SeNPs-treated group, reduced mononuclear cellular infiltration was observed compared to the HFD group; subgroup IIIA (NAS score 2) and subgroup IIIB (NAS score 1). This could be explained by their anti-inflammatory properties. SeNPs reduce the expression of key pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, primarily by inhibiting the NF- κ B signaling pathway, a central regulator of inflammation. SeNPs also promote a shift in macrophages to an anti-inflammatory phenotype, aiding in inflammation resolution and tissue repair.^[69]

The increased collagen deposition, pseudo-lobulation, and septa formation reported in the liver of group II HFD in both H&E and trichrome sections seems to be resolved following the

treatment with SeNPs, especially when combined with a normal diet, where the area percentage of the collagen fibers significantly decreased in morphometric studies reaching the control levels; subgroup IIIA (F1 score) and subgroup IIIB (F0 score). A recent study has shown that the antifibrotic effects of SeNPs are due to their significant role in decreasing almost all pro-fibrotic and pro-inflammatory genes. In addition, they have a role in inhibiting hepatic stellate cell activation and reducing transforming growth factor-beta (TGF- β) signaling, a key pathway in fibrogenesis. By modulating extracellular matrix deposition, SeNPs may prevent NAFLD from advancing to more severe stages.^[70]

These findings were further validated in our study where TGF- β expression levels were significantly lower in both SeNPs-treated subgroups compared to the HFD 10w group, particularly in the SeNPs + normal diet group.

Moreover, α -SMA is a key marker (and effector) of HSC trans-differentiation into contractile myofibroblasts during liver fibrosis was also affected where α -SMA expression showed significant increase in HFD-fed animals, with the highest levels observed after 10 weeks of HFD, reflecting progressive fibrogenesis. SeNPs administration significantly reduced α -SMA expression, with the most evident effect observed in the SeNPs + normal diet group, which was statistically indistinguishable from the control group.^[71]

The ultrastructural examination of group III revealed better results for most of the hepatocytes with restoration of the normal hepatic ultrastructure, thus confirming the LM findings in this group. Hepatocytes revealed fewer lipid droplets, preserved mitochondrial integrity, and well-maintained rER structure. It was noted that only a few hepatocytes revealed cytoplasmic rarefaction or appeared with clumped organelles.

No dilatations or proliferations of bile canaliculi were noticed in the treated group as compared to the HFD group. Instead, the bile canaliculi were observed with microvilli

protruding into their lumina and situated between two adjacent hepatocytes and tightly sealed by desmosomes, like the control groups.

In addition to the previous findings, Hepatic stellate cells (HSCs), also known as Ito cells or fat-storing cells, resided in the space of Disse. These cells play a central role in liver homeostasis and fibrosis. In their quiescent state, as seen in this group, HSCs store vitamin A in lipid droplets rich in retinoids. Upon chronic liver injury, including that seen in progressive NAFLD, HSCs undergo activation, transforming into myofibroblast-like cells that contribute to extracellular matrix deposition and fibrosis. A few myofibroblasts were still observed surrounded by some collagen fibers. During fibrosis resolution, activated hepatic stellate cells (HSCs) may undergo apoptosis or revert to an inactivated state.^[72, 73] which could account for the reduced number of HSCs observed in this group. Also, HPCs seen in SeNPs-treated livers, maybe as a result of reduced oxidative stress and improved metabolic homeostasis enhancing the activation and differentiation capacity of HPCs, creating a favorable microenvironment for regeneration over fibrosis.^[74, 75]

In light of the above findings, SeNPs were found to reverse the hepatic injury caused by HFD and promote liver regeneration by creating a restorative environment through their antioxidant and anti-inflammatory properties. They also supported DNA synthesis and cell cycle progression. These combined effects helped shift the liver from chronic injury and fibrosis toward healing and regenerative remodeling.

SeNPs effectively improved several metabolic and hepatic parameters in all treatment subgroups, restoring them to levels comparable to the control groups, particularly in subgroup IIIB (SeNPs+ diet modification). They significantly reduced elevated liver enzymes (ALT and AST), indicating reduced hepatocellular injury. They also improved the lipid profile by lowering TGs, LDL, and TC, while increasing HDL levels, aligning with previous findings on SeNPs' lipid-balancing effects.^[75, 76]

Also, SeNPs lowered fasting blood glucose, insulin levels, and HOMA-IR, suggesting enhanced insulin sensitivity. These metabolic improvements are attributed to SeNPs' antioxidant properties, as evidenced by reduced MDA levels and increased SOD activity and TAC, indicating reduced oxidative stress and improved redox balance in liver tissue, especially in subgroup IIIB.

All of these findings further support that the combination of SeNPs with dietary modulation appears to be more effective in alleviating NAFLD than SeNPs alone with HFD. While SeNPs exert significant antioxidant and anti-inflammatory effects, the persistence of a high-fat diet may counteract these benefits by sustaining metabolic stress and lipotoxicity.^[74]

Dietary modulation from HFD to normal diet targets the root cause of NAFLD by reducing lipid influx and improving insulin sensitivity.^[77] Combining the healthy diet and SeNPs appeared to act synergistically as SeNPs improve cellular redox balance, while dietary changes limit ongoing hepatic insult, thus creating an environment more conducive to hepatocyte regeneration and metabolic recovery.^[78] Therefore, integrated strategies that include both SeNPs administration and dietary correction offer a more beneficial and sustained approach to managing NAFLD and preventing its progression to NASH or cirrhosis.

Finally, a spontaneous recovery group was established in the current study to assess whether the effect of HFD could be reversed within 2 weeks. LM examination of this group revealed that they were still markedly affected with widespread hepatocellular damage. Persistence of the steatosis was confirmed by Oil-red-O and Osmium tetroxide stains evaluated by morphometric study (NAS score 2) with persistence of lobular inflammation (NAS score 3). Moreover, intense collagen staining in the trichrome-stained sections and its morphometric study showed no statistically significant difference in the collagen area percentage from the HFD group (Fibrosis stage 3). Electron microscopy confirmed the LM appearance of disruptive

hepatic parenchyma. Hepatocytes exhibited rarefied cytoplasm, ongoing presence of lipid droplets, and mitochondrial abnormalities.

There is obvious evidence that mild to moderate hepatic NAFLD and subsequent fibrosis is reversible upon the successful elimination of the underlying causes of chronic or recurrent liver inflammation. However, once extensive collagen crosslinking occurs, rendering the matrix resistant to collagenase-mediated degradation, the resulting fibrotic scar becomes highly stable and effectively irreversible.^[79] Our findings suggest that resolution might probably occur but required a longer duration or alternatively fibrosis may have reached an irreversible stage.

Biochemical results come hand in hand with the histological results, as liver function tests (ALT and AST) showed no significant improvement in this short period compared to the HFD group. In addition, no significant improvements were observed in insulin sensitivity (as assessed by HOMA-IR, fasting insulin levels, and fasting glucose. Lipid profile abnormalities were still evident, including persistent elevation of TGs and LDL, with low HDL and just some improvement in TC compared to the HFD group.

These findings agree with recent reports indicating that the resolution of HFD-induced injury requires a longer recovery period due to the persistence of hepatic lipid accumulation, chronic inflammation, and adipose tissue dysfunction.^[80, 81]

Additionally, HFD feeding induces sustained alterations in hepatic gene expression, including those involved in lipid and glucose metabolism, through mechanisms such as epigenetic modifications and transcriptional reprogramming, which may prolong metabolic impairment despite dietary normalization.^[81, 82]

In addition to persistent metabolic disturbances, the elevated levels of oxidative stress markers in this group such as tissue MDA, alongside reduced levels of SOD and TAC, following a two-week diet reversal from high-fat feeding, suggest that oxidative imbalance remains unresolved

in the short term. These findings are similar to previous studies reported that MDA reflects ongoing oxidative damage, which is a hallmark of HFD-induced hepatic injury.^[83]

The present work revealed, through a combination of biochemical, histological, and morphometric studies, that prolonged consumption of the HFD induces significant hepatic damage, including steatosis, inflammation, and fibrosis. However, administration of SeNPs demonstrated a protective effect by reducing lipid accumulation, preserving hepatocyte structure, and mitigating inflammatory changes with a better synergistic effect when combined with a normal diet. These findings suggest that SeNPs could serve as a promising therapeutic strategy for NAFLD. Further research is required to explore the underlying molecular mechanisms and the long-term efficacy of SeNPs in liver disease management.

5. CONCLUSION

In conclusion, the in-house prepared high-fat diet successfully established a rat model of NAFLD that reproduced key histopathological and biochemical features of the human disease, validating its use for evaluating selenium nanoparticles (SeNPs) as a therapeutic agent. SeNPs administration ameliorated HFD-induced hepatocellular injury, restored liver function parameters, and attenuated hepatic fibrosis, even when high-fat feeding continued. However, combining SeNPs treatment with dietary normalization produced the most favorable outcome, outperforming both continued HFD feeding with SeNPs treatment and dietary correction alone. These findings support SeNPs as a promising antioxidant-based adjunct therapy for NAFLD, particularly alongside dietary modification, though further studies are needed to clarify the underlying mechanisms and assess long-term safety.

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