Ultrastructural study on the hepatoprotective effect of selenium-loaded chitosan nanoparticles against silver nanoparticles-induced toxicity in the female albino rats.

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1. Abstract

Silver nanoparticles (Ag-NPs) are increasingly used in industrial and biological applications. There are growing worries regarding their toxicity to both humans and animals. Selenium-loaded chitosan nanoparticles (CS-SeNPs) have strong antioxidant properties via scavenging free radicals. The aim of this study is to assess the protective effect of CS-SeNPs against Ag-NPs' damaging effects on the hepatic tissue of adult female rats. Forty female albino rats were separated into four groups: group I (control) was given distilled water (0.5 ml/kg), group II was administered Ag-NPs (100 mg/kg), group III was co-exposed with Ag-NPs (100 mg/kg) and CS-SeNPs (0.5 mg/kg) and group IV received only CS-SeNPs (0.5 mg/kg) daily by oral gavage. After 60 days, rats were euthanized, then hepatic tissues were collected and fixed in 5% glutaraldehyde for transmission electron microscopy. Exposure to Ag-NPs induced severe degeneration of the hepatocytes, in the form of complete lysis and disappearance of the cytoplasmic organelles. While supplementation with CS-SeNPs exhibited a partial disappearance of most degenerative changes induced by Ag-NPs. In conclusion, Ag-NPs induce some destructive effects on the hepatic tissue and CS-SeNPs can ameliorate most of these harmful effects.

Keywords: Silver nanoparticles; Selenium-loaded chitosan nanoparticles; Hepatotoxicity; Ultrastructure.

2. Introduction

Nanoparticles (NPs) are made up of ultra-fine materials between 1 and 100 nm in size and have special characteristics that differentiate them from those of their bulk form **[1]**. Because of their incredibly small size, NPs have the capacity to penetrate the cells and cause damage. Silver nanoparticles (Ag-NPs) are frequently used in a wide variety of products, including wound dressings **[2]**, cosmetics, household goods, medical products, and as a potential animal feed additive instead of antibiotics **[3, 4]**. There is a high potential risk of rapid oral, transdermal, or inhalation exposure due to the extensive usage of nano silver in consumer products **[5]**. Additionally, Ag-NPs may be released into the ecosystem with wastewater contaminating natural water systems, which negatively impacted on the aquatic organisms **[6]**. Many investigations have demonstrated

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that Ag-NPs have a negative impact on the mitochondrial respiratory pathway, resulting in a significant release of reactive oxygen species (ROS) with impairment of ATP synthesis **[7]**. The liver is the main target organ of Ag-NPs' toxic effects through all the exposure routes **[8]** inducing ROS generation, peroxidation of the cellular macromolecules, and inflammation of the hepatic tissue **[9]**.

Selenium (Se) is a crucial micronutrient for both humans and animals due to its anti-inflammatory and antioxidant properties through the activation of selenoenzymes such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR) preventing oxidative damage to body tissues [10]. Se deficiency is linked to many diseases inducing liver damage. Se-NPs have ability to protect animals from toxins or pathogens that cause liver damage suggesting their hepatoprotective properties **[11]**.

Chitosan (CS), has biological properties that attract a lot of attention, including antibacterial, anti-aging, antioxidant, immune-enhancing, and anticancer activities **[12]**. Additionally, due to its low toxicity and capacity to increase drug absorption and bio-adhesion, it is frequently used as a carrier for a wide variety of drugs **[13]**. Therefore, our study aimed to investigate the potential protective effect of CS-SeNPs via their antioxidant and anti-inflammatory activities against the toxicity of Ag-NPs on the ultrastructure of the hepatic tissue.

3. Materials and Methods

3.1. Animals:

Forty adult female Albino rats were taken from the breeding unit of the Animal Health Research Center, Dokki, Egypt. Rats were kept in plastic cages and acclimated to the laboratory environment (illuminated room with controlled humidity (50±10%) and temperature (25±5°C)) for 7 days before the beginning of the study. Rats were given a standard laboratory rat diet and unlimited access to water. The experimental procedure was fulfilled with the instructions for animal experiments which were approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, Cairo University (Vet. Cu. IACUC, No. 8-03-2022-435).

3.2. Silver nanoparticles (Ag-NPs) and Selenium-loaded chitosan nanoparticles (CS-SeNPs) synthesis:

Chemical reduction method was used to produce silver nanoparticles **[14, 15]**. Briefly, 200 ml of 0.01 M sodium borohydride (Loba Chemie Pvt. Ltd.®) and 100 ml of 0.14 M AgNO3 (Loba Chemie Pvt. Ltd.®; India) solution were mixed with 10% polyvinyl pyrrolidone (PVP) (Loba Chemie Pvt. Ltd®). The color of the silver solution gradually turned greyish, representing the reduction of the silver ions to nanoparticles.

Selenium-loaded chitosan nanoparticles were synthesized by the ionotropic gelation procedure **[16, 17].** In brief, chitosan (Mw100:300 KDa, deacetylated degree 90%; ACROS

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ORGANICS®; UK) solution (2.5 mg/mL) was produced by combining chitosan with diluted acetic acid (1% (v/v)) then stirring until chitosan was completely dissolved. The pH was then corrected to 4.6 using 4 M NaOH. Overnight, the mixture was stirred at room temperature. The next day, the chitosan solution was stirred at 500 rpm while sodium selenite (SDFCL®; India) aqueous solution (0.5 mg/mL) was added dropwise. The resulting chitosan/selenium solution was stirred for 4 hours at room temperature. Next, the weight ratio of chitosan to tripolyphosphate (CS to TPP) was adjusted to 3:1 by adding the tripolyphosphate (TPP) aqueous solution (1 mg/mL) dropwise to the chitosan/selenium mixture and stirring for 4 hours. The nanoparticles (NPs) solution was then washed with distilled water and centrifuged at 13, 000 g for 20 min.

3.3. Nanoparticles (NPs) characterization: morphology and size:

Nanoparticles' diameter was established via scanning electron microscopy (SEM) (XL-30 ESEMFEG SEM, FEI Company, USA). Using image analysis software, the average diameters of 500 particles were assessed from SEM images (ImageJ, National Institutes of Health, version 1.5a, ImageJ.nih.gov). Zeta potential analysis was done to determine the surface charge of the hydrated NPs. To evaluate the encapsulation efficiency, 1 mg/ml of CS-Se NPs water solution was filtered by using a 0.2 μm syringe filter. 2 ml of 0.1% (w/v) 2,3-diaminonaphthalene (DAN) in 0.1 M HCl solution and 2 ml of 0.05 M EDTA were mixed with the filtrate on plate stirrer at 60˚C. The reaction's product, piaselenol, was isolated in 4 mL of cyclohexane and measured in triplicate using a microplate spectrophotometer at 378 nm. From a standard curve of known selenium concentrations, the amount of selenium was determined. The following equation was used to calculate encapsulation efficiency (EE). In vitro, as previously discussed, the selenium release profile was established **[18, 19]**. In brief, to make a 1 mg/mL NPs solution, CS-Se-NPs were first suspended in phosphate buffer saline (1 PBS, pH 7.4) and then put into a shaker for gentle agitation at 37 °C. Samples were taken at various intervals (1, 2, 4, 8, and 24 hours), and the amount of Selenium released from the capsule was determined as previously mentioned.

$$\text{EE\%} = \frac{\text{Amount of encapsulated selenium}}{\text{Total amount of selenium}} \times 100$$

3.4. Experimental design:

After the acclimatization interval (7 days), rats were separated into four groups (10 rats each) as follows: group I (Control) was given only distilled water (0.5 mL/kg), group II (Ag-NPs) received Ag-NPs (100 mg/kg) **[20]**. group III (Ag-NPs + CS-SeNPs) received Ag-NPs (100 mg/kg) with CS-SeNPs (0.5mg/kg) **[21]**, and group IV (CS-SeNPs) was administered CS-SeNPs (0.5 mg/kg)

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daily for 60 days by oral gavage. The body weight of rats was measured and documented weekly.

3.5. Collection of samples:

At the end of the experiment, rats were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) then were sacrificed by cervical decapitation. Liver specimens were collected and fixed in 5% glutaraldehyde for transmission electron microscopic examination.

3.6. Transmission Electron Microscopy (TEM):

Liver tissue was dissected into approximately 1 mm^3 fixed in 5% glutaraldehyde in phosphate buffer (pH 7.2–7.4) for 3 hr. **[22]**. The specimens were processed according to **[23]**. Ultrathin sections were examined by TEM-109 (SEO company) in the Faculty of Agriculture, Cairo university, Electron Microscopy Unit, Egypt.

4. Results

4.1. Nanoparticle Characterization:

The morphology of silver nanoparticles (Ag-NPs) and selenium-loaded chitosan (CS-Se NPs). Ag-NPs and CS-SeNPs showed spherical morphology with average diameters measured through SEM images were 44.6 ± 8.5 nm and 241.6 ± 26.8, respectively. The zeta potentials of Ag-NPs and CS-SeNPs were -13.2 ± 0.4 mV and +42.6 ± 0.5 mV, respectively **(Figure.1)**. Chitosan demonstrated high encapsulation of Se with an EE of 87.8 ± 4.4%. The selenium surged release at the first 4 hours of incubation (84.7 ± 1.7 %) then followed by gradual release till the end of the experiment at 24 hours (**Figure**.**2)**.

4.2. Transmission electron microscopical (TEM) examination:

Ultrastructural examination of liver sections of the control group **(Fig.3A)** and CS-SeNPs **(Fig.3 B)** revealed normal morphology of the hepatocyte with its normal central spherical euchromatic nucleus having normal prominent nucleolus. Moreover, the nucleus had a normal intact nuclear membrane and evenly distributed chromatin. Hepatocyte's cytoplasm contained normal organelles as numerous mitochondria, well developed rough endoplasmic reticulum (RER) with its bounded ribosomes and smooth endoplasmic reticulum (SER). Normal Kupffer cell in the wall of the blood sinusoid was also observed.

In contrast, liver sections from Ag-NPs exposed group revealed severely degenerated hepatocytes, in the form of complete lysis and disappearance of the cytoplasmic organelles with disintegration of the cell membrane. Furthermore, the nuclear membrane appeared thin and irregular with loss of the chromatin content which indicated chromatolysis. In addition to, the nucleoli completely disintegrated and disappeared **(Fig.3C)**.

While co-administration of Ag-NPs with CS-SeNPs revealed partial

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recovery of the hepatic parenchyma in comparison with Ag-NPs exposed group. Most hepatocytes appeared nearly normal restoring their typical polygonal shape with their central spherical euchromatic nuclei. The nucleus appeared surrounded by a normal intact nuclear membrane with a normal distribution of nuclear chromatin. The nucleoli appeared clear with normal shape and size. Kupffer cells also appeared nearly normal in the blood sinusoids. Only some hepatocytes appeared with multiple cytoplasmic vacuoles were observed. **(Fig.3D)**.

5. Discussion

Silver nanoparticles (Ag-NPs) are widely used in a wide variety of fields, including medicine and industery, making their discharge into the environment uncontrolled. Therefore, potential health risks cannot be disregarded **[24, 25]**. Selenium nanoparticles (Se-NPs) protect the body against metal toxicity, as revealed by **[26]**. Moreover, cellular enzymes and nucleic acids are protected from reactive oxygen species (ROS) damage by selenium **[27]**.

Chitosan is resistant to pepsin and pancreatic enzymes, giving chitosan-coated nanoparticles high oral bioactivity **[28]**. Furthermore, chitosan nanoparticles are used in the drug delivery system having the benefit of controlled drug release, which increases drug solubility and stability, intensifies efficacy, and lowers their toxicity **[29].**

In our study, selenium was loaded on chitosan nanoparticles (CS-SeNPs), and the results show a small average size of CS-SeNPs due to the ionic gelation approach that was used for their synthesis **[30]**. Moreover, the positive zeta potential charge of the CS-SeNPs maintains the stability of the nanoparticles, reduces the probability of aggregation, and enhances electrostatic interaction with the total negative charge of the cell membrane **[31].** The positive zeta potential values may result from residual amino groups in the chitosan molecules that are entangled with the surface of the nanoparticles values **[32]**. Chitosan nanoparticles also exhibited high selenium loading, with 88.6 4.6%. An earlier study of **[33]** who found that the ratio of chitosan to tripolyphosphate (TPP) during the synthesis of nanoparticles determines the ability of chitosan to encapsulate compounds. A larger TPP content during manufacturing increased chitosan's loading efficiency. According to the releasing profile of the CS-SeNPs, Se was released in a burst during the first four hours of incubation then gradually through the following hours. These results could be explained by the distribution of Se close to the surface of chitosan nanoparticles and the large surface area of these particles. Consequently, Se desorption from the surface of the nanoparticles could be the cause of the initial burst release **[34]**. Following the burst release period, the release showed a gradual pattern that caused by Se exposure from the degradation of the polymer **[35]**.

The ultrastructural examination of the liver tissue of the rats exposed to Ag-NPs

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revealed severe degeneration of the hepatocytes with complete lysis of their cytoplasmic organelles. Moreover, irregularity and thinning of the nuclear membrane was also observed that is in harmony with **[36]** and **[37]** who reported that Ag-NPs cause degeneration and apoptosis of the hepatocytes, with irregularity in the nuclear membrane. This may be accounted for the excessive ROS release that led to oxidative stress, which initiated direct or indirect chain reactions with cellular macromolecules like DNA, protein, carbohydrates, and lipid, impairing the crucial cellular functions and potentially resulting in a significant cell death **[38, 39]**. **[40]** reported that Ag-NPs toxicity mainly affects the mitochondria by altering their membrane permeability and disrupting their respiratory chain, which causes ROS release, necrosis, and apoptosis.

While CS-SeNPs showed partial recovery of the hepatic parenchyma that appeared in the form of restoring the normal polygonal shape of most hepatocytes with their spherical and euchromatic nuclei with restoring the normal distribution of the nuclear chromatin. These results in consistent with the previous study of **[41]** who found that the nucleus began to restore its normal shape and the nucleolus became clear in liver sections of SeNPs exposed rats. That may be explained by the enhancement action of Se-NPs, which raises the amounts of the antioxidant enzymes and consequently repairs oxidative damage of the cells. Additionally, chitosan displayed remarkable antioxidant activity through a potent scavenging action by donating hydrogen atoms **[42].** According to various studies, Se-NPs alone or in combination with vitamins may protect animals from the hepatic damage induced by toxic substances such as acetaminophen **[43]**, carbon tetrachloride **[44]**, and acrylamide **[45]**.

6. Conclusion

In conclusion, despite the widespread use of Ag-NPs as possible animal feed additives and in the delivery of some drugs, public concerns regarding their toxicity had grown. We demonstrated that the novel CS-SeNPs mixture was highly effective in preventing Ag-NPs-induced hepatic toxicity that appeared in the form of degenerative alterations in the hepatic tissue. However, most of these negative effects induced by Ag-NPs were attenuated by co-supplementation with CS-SeNPs. Additionally, selenium's action is improved by chitosan coating. Suggesting that CS-SeNPs supplementation may offer protection against the hepatic toxicity induced by Ag-NPs.

Conflict of interest

The authors state no conflicts of interest.

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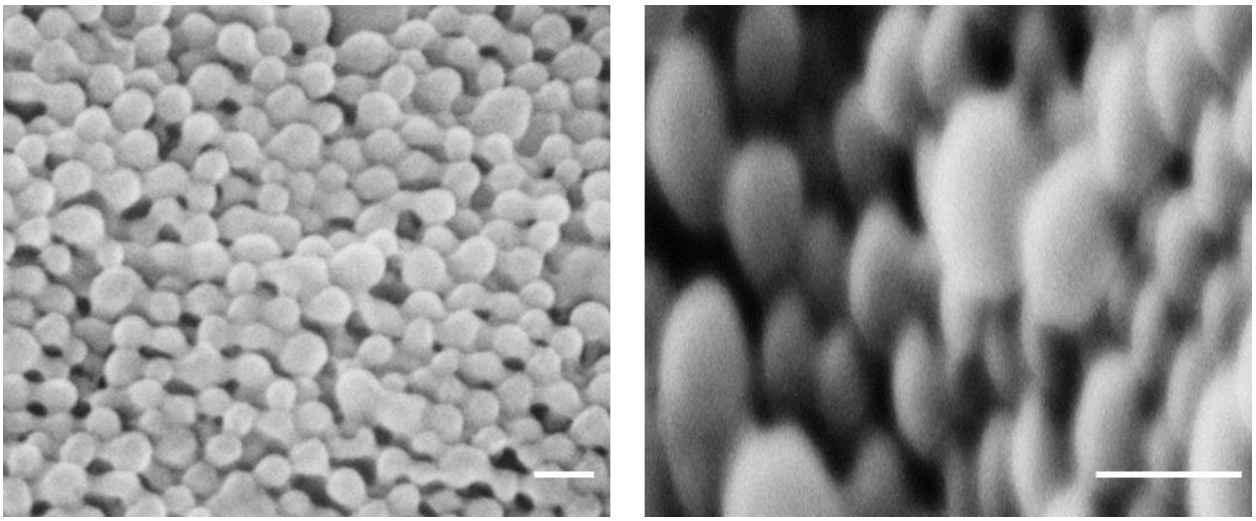

Figure (1): SEM images of (A) Ag-NPs and (B) CS-Se NPs. Scale bars represent 200 nm and 1 μm, respectively.

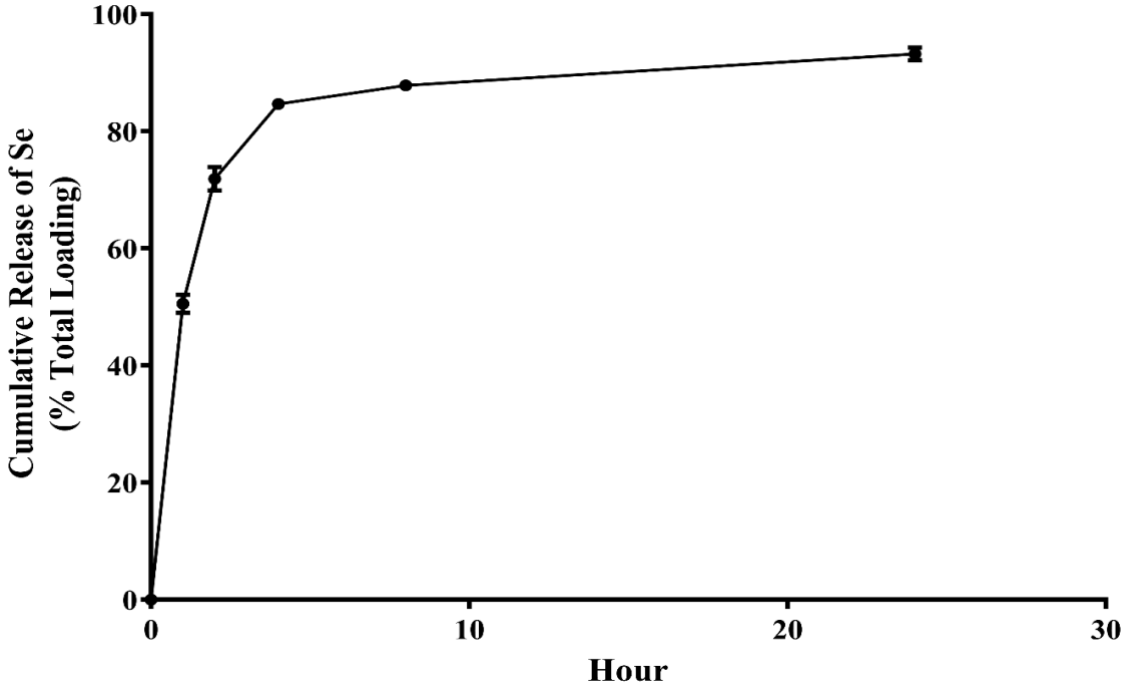

Figure (2): Cumulative release of selenium from chitosan NPs over 24 hr. period. Data are presented as (mean ± SD, n=3).

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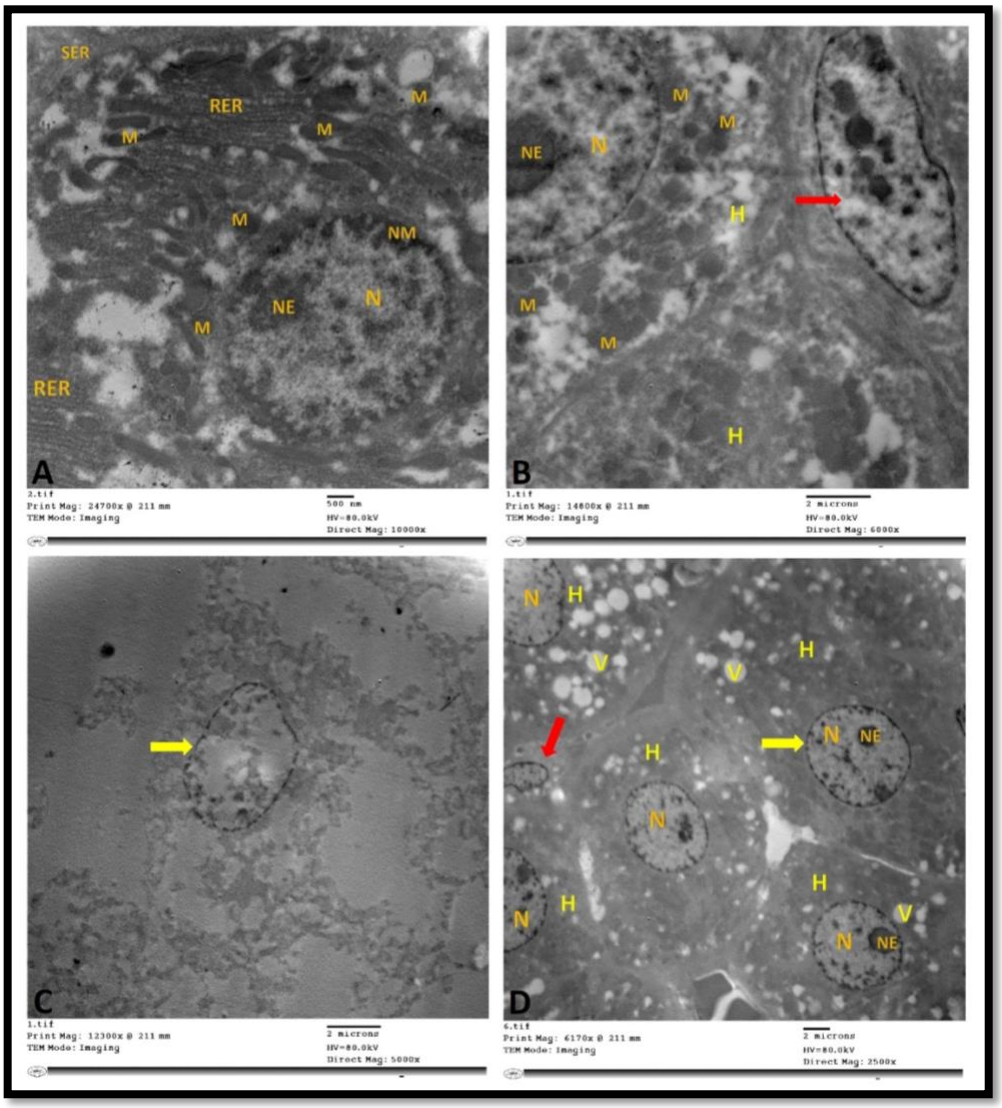

Figure (3): TEM micrograph of albino rats' liver sections of **A.** control group: showing normal hepatocyte with its euchromatic nucleus (N) having prominent nucleolus (NE), evenly distributed chromatin and intact nuclear membrane (NM). Normal mitochondria (M), rough endoplasmic reticulum (RER) with its bounded ribosome and smooth endoplasmic reticulum (SER). X 10000 **B.** CS-SeNPs administered group: showing hepatocytes (H) having normal nuclear morphology (N) with its prominent nucleolus (NE) and normal cytoplasm with numerous mitochondria (M). Normal kupffer cell (red arrow) X 6000. **C.** Ag-NPs exposed group showing severly degenerated hepatocyte with complete lysis of its organelles, disappearence of the nucleolus and chromatolysis with thin irregular nuclear membrane (arrow) X 5000. **D.** Ag-NPs and CS-SeNPs co-administered group showing nearly normal hepatocytes (H) that appeared with their normal polygonal shape and structure. Some cytoplasmic vacuoles are seen in some hepatocytes (V). The nucleus appeared nearly normal (N) with intact nuclear membrane (yellow arrow) and prominent nucleolus (NE). Nearly normal kupffer cell appeared in the hepatic sinusoid (red arrow) was also observed X2500. (Uranyl acetate and lead citrate stain).

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