Trop J Nat Prod Res, September 2025; 9(9): 4137 - 4142 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Chitosan Nanoparticles as a Therapeutic Agent for Mitigating Paracetamol-Induced Liver Damage in White Male Albino Rats

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ARTICLE INFO

Article history:
Received 07 February 2025
Revised 02 March 2025
Accepted 18 September 2025
Published online 01 October 2025

ABSTRACT

High paracetamol doses can cause liver damage, but chitosan nanoparticles (ChNPs), known for their biocompatibility, biodegradability, anti-inflammatory, and antioxidant properties, may offer potential therapeutic benefits. This study investigated the protective effects of ChNPs in mitigating paracetamol-induced liver damage in white male albino rats. Chitosan nanoparticles were synthesized and characterized using scanning electron microscopy, atomic force microscopy, and UV-Vis spectroscopy. Sixty adult male white albino rats (200–250 g) were randomly assigned to six groups and treated orally for three months. Groups received distilled water (Group I), 15 mg/kg ChNPs (Group II), 500 mg/kg (Group III), or 1000 mg/kg (Group IV) paracetamol or combinations of 500 mg/kg (Group V) or 500 mg/kg (Group VI) paracetamol with 15 mg/kg ChNPs. Liver tissues were histologically examined, and DNA damage was assessed. The results showed that ChNPs (43–80 nm) exhibited smooth, spherical morphology with improved dispersion. Prolonged oral administration of paracetamol at both doses induced significant histopathological changes, including fibrosis, congestion, and inflammatory infiltration in liver tissues. Co-administration with ChNPs preserved liver architecture, reducing morphological abnormalities. The comet assay showed significant DNA strand breaks in paracetamol-treated rats, indicated by increased tail DNA% and tail length. Chitosan nanoparticles mitigated DNA damage, likely due to their antioxidant and anti-inflammatory properties. The study's findings revealed that ChNPs exhibited a protective effect against paracetamol-induced hepatotoxicity, mitigating both histopathological and genetic alterations. These findings underscore the potential therapeutic application of ChNPs in preventing drug-induced liver damage and support their broader use in nanomedicine.

Keywords: Chitosan nanoparticles, Paracetamol, Comet assay, Hepatotoxicity, Microemulsion method

Introduction

Paracetamol is an analgesic medication that does not have anti-inflammatory properties. It is commonly used to relieve muscle and joint pain, symptoms of cold and flu, common headaches, and fever.[1] Although paracetamol is usually regarded as safe, an acute excess can cause liver, kidney, and brain damage that could be fatal. In rare cases, an organ may sustain damage even at a therapeutic dosage.[2] Paracetamol is mainly processed in the liver, where it is converted into various metabolites. Most of these metabolites are non-toxic and inactive, and they are eventually eliminated by the kidneys. However, in the case of an overdose, N-acetyl-p-benzoquinone imine (NAPQI), a hazardous chemical, is produced during the metabolism of paracetamol. The accumulation of excess NAPQI leads to liver damage by triggering the production of free radicals and oxidative stress.[1,2] Nanotechnology has gained significant attention since the 1980s and has been applied in various design fields, particularly in biomedical applications, such as controlled drug delivery and tissue engineering.[3]

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Citation: Al-Bhadly O, Mnati IM. Chitosan nanoparticles as a therapeutic agent for mitigating paracetamol-induced liver damage in white male albino rats. Trop. J. Nat. Prod. Res. 2025; 9(9) 4137 – 4142 https://doi.org/10.26538/tjnpr/v9i9.9

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Among nanomaterials, nanoparticles have contributed significantly to advancements in the biomedical field, particularly in the diagnosis and treatment of diabetes, infections, inflammation, and cancer.[4]Chitosan can be derived from various natural sources after the deacetylation of chitin, including fungal cell walls, mushrooms, insects, and marine crustaceans, such as crabs, krill, shrimp, and cuttlefish.[5] It has a complex double-helix structure and contains various active groups, including hydroxyl and amino groups, which exhibit high reactivity. To achieve unique physical and chemical properties, these groups can be chemically modified. Chitosan has attracted significant attention due to its exceptional properties and broad range of potential applications. The number of publications related to this polymer continues to grow each year.[6] Its enhanced structural and functional properties, combined with its biocompatibility, biodegradability, and non-toxicity, have made chitosan and its nanoparticles a focal point for researchers in various fields.[7] One of the primary benefits of chitosan is its capacity to control and slow the release of drugs, improving their stability and solubility, and reducing toxicity.[8] Based on the information presented, the United States Food and Drug Administration (FDA) has classified chitosan as generally safe, leading to its widespread use in biomedical applications.[7,8]These include its role as a binder material for drug tablet preparation, immunoenhancing and antitumor activities,[9] wound healing, pharmaceuticals, cosmetics, antibacterial and anti-inflammatory effects, anti-apoptotic properties, and suppression of oxidative stress.[10] Presently, chitosan nanoparticles have attracted considerable interest in nanomedicine due to their ability to enhance bioavailability, increase specificity and sensitivity, and reduce pharmacological toxicity.[11]

The present study investigated the protective effects of chitosan nanoparticles, known for their biocompatibility, biodegradability, anti-inflammatory, and antioxidant properties, in mitigating liver damage caused by prolonged exposure to maximum daily and toxic doses of paracetamol. The study explored chitosan nanoparticles as a hepatoprotective agent against paracetamol-induced liver damage, evaluating their efficacy in reducing histopathological and genetic alterations.

Materials and Methods

Sources of animals

In this investigation, 60 adult male white albino rats weighing between 200 and 250 g were obtained from the Biotechnology Research Center at AL-Nahrain University. They were fed a standard commercial pellet diet and provided with unlimited water while housed in polypropylene cages under controlled environmental conditions (12-hour light/dark cycle and a temperature of 25°C).

Ethical approval

Ethical approval for the study was obtained from the Ethics Committee of the Collage of Education for Pure Science (Ibn Al- Haitham), University of Baghdad, Baghdad, Iraq (Approval No.: EC 48, Dated on: December 12, 2024).

Chitosan nanoparticle preparation

The microemulsion method was used to prepare chitosan nanoparticles in this study. Two separate phases (organic and solution) were prepared. The organic phase was prepared by dissolving the surfactant Tween 80 (polysorbate 80) and glutaraldehyde in n-hexane. The mixture was stirred continuously at room temperature for 30 minutes. In the second phase, the aqueous phase was prepared by dissolving glacial acetic acid and chitosan in deionized water and stirring at room temperature for 30 minutes. The organic solution was progressively added to the aqueous solution, which was agitated overnight to allow complete crosslinking of the free amine groups of chitosan and glutaraldehyde. After confirming that the crosslinking process was complete, the organic solvent was evaporated at low pressure with a high vacuum. The resultant nanoparticles were then centrifuged at 14,000 rpm. Finally, the precipitated particles were dried by placing them in an oven at 45°C.

Characterization of nanostructure and microstructure chitosan

The morphology and particle size of the samples were analyzed using the field emission scanning electron microscopy (FESEM; Czech Republic). The materials' 3D surface topography was examined using atomic force microscopy (AFM). Ultraviolet-visible (UV-Vis) spectrophotometry was performed using a Shimadzu 3600 UV-Vis absorption spectrophotometer (Japan) to evaluate the photocatalytic optical absorption properties.

Experimental grouping and treatments

This study was conducted at the Biotechnology Research Center of Al-Nahrain University. The rats were randomly assigned to six groups, each consisting of ten individuals. Over a period of three consecutive months, each group of rats was treated daily via oral administration. The control group received distilled water (Group 1), while another group was given chitosan nanoparticles at a concentration of 15 mg/kg (Group II). Separate groups were administered paracetamol at concentrations of 500 mg/kg (Group III) and 1000 mg/kg (Group IV). Additionally, two groups received a combination treatment: one with 500 mg/kg of paracetamol alongside 15 mg/kg of chitosan nanoparticles (Group V), and the other with 1000 mg/kg of paracetamol combined with the same concentration of chitosan nanoparticles (Group 6). The maximum daily dose of paracetamol for an adult human is 4,000 mg, while the high toxic dose ranges from 10,000 to 15,000 mg.[12] Based on the study of Al-Doaiss,[13] the acceptable dose for rats was established by dividing the maximum daily dose for an adult human (weighing approximately 55-75 kg) and multiplying by a factor of seven to account for the difference in body surface area between humans and rats.

Preparation of tissues for histopathological examination

Rat liver tissues were collected immediately after sacrifice, sliced, and preserved in 10% formalin. Tissue slices were prepared and stained using standard protocols, including Hematoxylin and Eosin (H&E) and Masson's trichrome staining. The stained slices from all groups were then examined using a light microscope at 10x.

Quantification of DNA damage with the comet assay

DNA damage was assessed using the comet assay.[14] In brief, a liver tissue homogenate was mixed with 20 µL low melting point agarose (LMPA) and placed onto slides pre-coated with normal melting point agarose (NMPA). The slides were then incubated for one hour on ice-cold lysis buffer (pH 10) containing 100 mM Na_2EDTA, 2500 mM NaCl, 1% Triton X100, 0.01 M Tris, and 10% DMSO. After lysis, electrophoresis was performed in an alkaline buffer (pH 13, 1 mM Na_2 EDTA) at 38 V and 294 mA. Following electrophoresis, the slides were neutralized with 400 mM Tris-HCl (pH 7.5) for 5 minutes, fixed in 96% ethanol, and stained with SYBR Green for 30 minutes. A cover slip was placed over the slide, and DNA damage was visualized using fluorescence microscopy (Movel Scientific Instrument Co., Ltd., China). All parameters were calculated using the Comet Assay Software Project (CASP) Lab (version 1.2.3b).

Statistical analysis

The values of the examined parameters are expressed as mean ± standard deviation. Differences between the means were analyzed using one-way analysis of variance (ANOVA) in Statistical Package for Social Sciences (SPSS; version 23.1) software. A p-value of ≤ 0.05 was considered statistically significant.

Results and Discussion

Characteristics of chitosan nanoparticles

Chitosan powder and prepared chitosan nanoparticles were characterized by FESEM, as shown in Figures 1A and B. The morphological structure of chitosan powder exhibited an irregular shape, large particle size, and an agglomerated state. In contrast, chitosan nanoparticles displayed a spherical shape, homogeneous structure, smooth surface, and good dispersion, with a particle diameter ranging from 43 to 80 nm. Additionally, the AFM was used in this study to quantitatively measure the root mean square (RMS) roughness of the particles. As depicted in Figures 2A and B, the RMS roughness for chitosan powder was observed to be 18.6 nm, while for chitosan nanoparticles, it was reduced to 1.15 nm, indicating a smoother surface for the nanoparticles. The optical properties of chitosan powder and prepared chitosan nanoparticles were analyzed using UV-Vis spectroscopy, as shown in Figures 3A and B. The UV-Vis spectra revealed that chitosan powder exhibited an absorption peak at 290 nm. In contrast, chitosan nanoparticles displayed a shift in the absorption maxima to a lower wavelength of 270 nm. Moreover, the UV-Vis spectrum of chitosan powder showed broad absorption band intensity, while the prepared chitosan nanoparticles exhibited a sharper intensity, reflecting the change in their structural properties.

In this study, significant morphological and structural differences between chitosan powder and chitosan nanoparticles were observed through SEM and AFM analyses. Scanning electron microscopy images revealed that chitosan nanoparticles exhibited an almost spherical shape, homogeneity, smooth surface, and good dispersion, with particle sizes ranging from 43 to 80 nm. These findings align with previous studies that report similar nanoparticle characteristics.[15] Atomic force microscopy analysis revealed a substantial reduction in RMS roughness, from 18.6 nm for chitosan to 1.15 nm for chitosan nanoparticles. This reduction is likely due to the smaller particle size of the nanoparticles, as also confirmed by the observed decrease in particle size.[16]

The increase in particle density, with a higher number of particles per mm^2, further supports the successful synthesis of smaller and more uniform nanoparticles, which is consistent with earlier reports.[11] UV-Vis spectrophotometric analysis showed a shift in the absorption maxima to a lower wavelength of 270 nm for chitosan nanoparticles,

suggesting successful formation of nanoparticles through the microemulsion process. This shift is associated with the CO group and confirms that the high-energy emulsification process was effective in generating chitosan nanoparticles. These results are in agreement with previous studies where similar findings were reported regarding the successful synthesis of chitosan nanoparticles.[17] Overall, the findings demonstrate that the microemulsion method effectively produced chitosan nanoparticles with desirable size, surface properties, and dispersion characteristics.

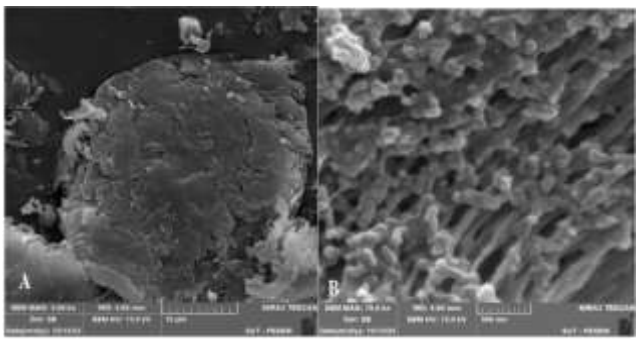

Figure 1: Field emission scanning electron microscopy images of studied substances. A: chitosan; B: chitosan nanoparticles.

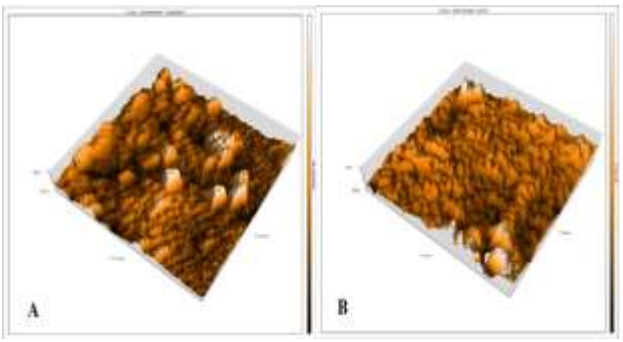

Figure 2: Atomic force microscopy images of studied substances. A: chitosan; B: chitosan nanoparticles.

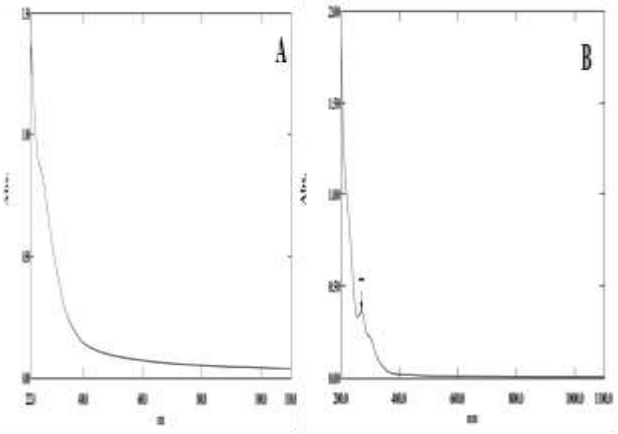

Figure 3: Ultraviolet-visible spectrophotometric analysis of studied substances. A: chitosan; B: chitosan nanoparticles.

Histopathological changes in rat liver

As shown in Figure 4A, the histological section of the rat liver from the control group (Group I) exhibited a normal architecture, including hepatocytes, the central vein, sinusoids, and the portal area. In Group II, the results showed no changes in the liver histology of rats administered only chitosan nanoparticles, and the findings are similar to those of the control group (Figure 4B). In animals receiving paracetamol at a concentration of 500 mg/kg (Group III), the hepatic sinusoids exhibited mild dilation, with small hemorrhagic areas and infiltration of inflammatory cells in the liver parenchyma. Additionally, there was minimal dilation and central vein congestion (Figure 4C). Additionally, severe congestion of the portal vein, along with inflammatory cell infiltration and early fibrosis, was observed around the portal area and within the liver parenchyma, as depicted in Figures 4D and E.

The results (Figure 4F) showed that animals receiving paracetamol at a concentration of 1000 mg/kg (Group IV) exhibited more pronounced morphological changes compared to Group III, including dilated sinusoids and highly dilated central and portal veins with intense congestion. There was also a significant amount of inflammatory cell infiltration in the liver parenchyma and surrounding the portal region. More fibrosis was observed both in the liver parenchyma and around the portal region. One of the significant changes at this concentration is the beginning of hyalinization (Figures 4G, H, and I). Additionally, marked parenchymal necrosis and lipid degeneration were observed in the liver parenchyma, along with an increased number of Kupffer cells (Figure 4J). Group V rats (administered 500 mg/kg of paracetamol and 15 mg/kg of chitosan nanoparticles) demonstrated the hepatocyte parenchyma preservation with only slight morphological changes, in contrast to the groups that received only paracetamol. The rats in this group demonstrated very minimal lymphoid cell infiltration within the liver parenchyma (Figure 4K). The rats in Group VI that were administered a combination of 1000 mg/kg paracetamol and 15 mg/kg chitosan nanoparticles exhibited significant protection against these toxicants. This was evidenced by the formation of a normal hepatic sinusoid. Additionally, there was an absence of effaced architecture, including congestion in the central and portal veins, apoptotic cells, and fibrosis. However, minimal accumulation of inflammatory cells and erythrocytes was observed (Figures 4L and M).

Histological investigations revealed that administering paracetamol at the maximum daily dose and a high toxic dose for three consecutive months caused varying degrees of liver tissue damage in the treated rats. Histopathological assessments of liver tissues confirmed that paracetamol triggers liver fibrosis, congestion, and inflammatory cell infiltration, revealing notably disorganized hepatic structures. The histological alterations observed in this study align with findings reported by other researchers.[18] Paracetamol causes liver damage through its poisonous metabolite, NAPQI, which is ordinarily detoxified by hepatic glutathione (GSH), forming a paracetamol-GSH conjugate.[19] However, in cases of paracetamol overdose, hepatic GSH levels are depleted, leading to a significant accumulation of NAPQI in the liver. This toxic metabolite interacts with various cellular proteins, disrupting their functions, causing cellular damage, and ultimately resulting in organ failure.[20] Extra NAPQI also promotes oxidative stress and forms covalent bonds with liver proteins.[21] Meanwhile, the exact molecular mechanisms of paracetamol-induced hepatotoxicity remain unclear. Mitochondria are believed to play a main role in liver cell death triggered through paracetamol toxicity.[22]

The findings of this study revealed that chitosan nanoparticles provided substantial protection against paracetamol-induced liver damage, as confirmed by histological examination of the liver tissues. Recent research has increasingly emphasized the potential of biocompatible natural compounds and biodegradable polymers as drug delivery systems or regulators to mitigate tissue damage caused by toxic substances. Among these, chitosan derived from the deacetylation process of chitin has emerged as one of the most extensively utilized biopolymers in the pharmaceutical industry.[23] Chitosan has shown considerable potential anti-inflammatory capabilities by interacting with immune cells, modulating immune responses, and regulating cytokines production. These properties make it a promising candidate for managing conditions associated with chronic inflammation.[24] Notably, it exhibits remarkable antioxidant activity, primarily through its potent scavenging action by donating hydrogen atoms.[25] This property positions chitosan as a versatile agent with therapeutic applications. Chitosan nanoparticles have gained popularity in drug

delivery systems because of their capacity to allow for regulated drug release. This technique improves drug solubility and stability, increases efficacy, and decreases toxicity.[25] As a polymeric drug delivery method, chitosan nanoparticles are particularly advantageous for oral drug delivery and enhancing intestinal drug absorption. Their positive charge facilitates strong attachment to cellular surfaces, thereby increasing the likelihood of cellular uptake, which makes them an excellent choice for multiple therapeutic applications.[26] These results highlight how adaptable chitosan nanoparticles are as a strong foundation for therapeutic uses and drug delivery.

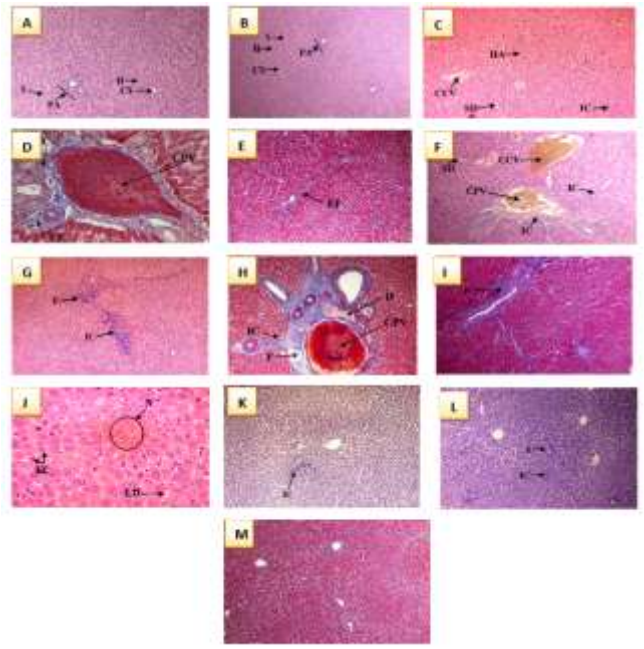

Figure 4: Histopathological micrographs of liver cells of rats treated with paracetamol only or combination with chitosan nanoparticles. A: Group I showing normal microscopic structure including hepatocytes (H), central vein (CV), sinusoid (S) and portal area (PA) (H&E 10X); B: Group II showing normal microscopic structure including hepatocytes (H), central vein (CV), sinusoids (S), portal area (PA) (H&E 10X); C: Group III showing sinusoid dilated (SD), hemorrhagic area in the liver parenchyma (HA), inflammatory cell (IC), congestion of the central vein (CCV) (H&E 10X); D: The portal area of the Group III showing congestion of the portal vein (CPV), inflammatory cell (IC), early fibrosis (EF) Masson's trichrome (40x); E: Group III showing early fibrosis in the liver parenchyma (EF) Masson's trichrome (10x); F: Group IV showing sinusoid dilated (SD) congestion of the central vein (CCV), congestion of the portal vein (CPV), inflammatory cell (IC) (H&E 40x); G: Group IV showing massive inflammatory cell (IC), fibrosis (F) in the liver parenchyma (H&E 10x); H: Group IV showing congestion of the portal vein (CPV), inflammatory cell (IC), fibrosis (F), Hyalinization (H) Masson's trichrome (40x); I: Group IV showing large area of the fibrosis (F) in the liver parenchyma Masson's trichrome (10x); J: Group IV showing large area of the hepatocyte necrosis (N), lipid degeneration (LD), Kupffer cell (KC) (H&E 40x); K: Group V showing an almost normal microscopic structure, with only a slight presence of inflammatory cells (IC) (H&E 10x); L: Group VI showing an almost normal microscopic structure, with only a slight presence of inflammatory cells (IC), erythrocytes (E) (H&E 10x);. M: Group VI showing an almost normal microscopic structure and absence of effaced architecture including, congestion in the central and portal veins and fibrosis Masson's trichrome (10x).

Effects of chitosan nanoparticles on DNA strand break

DNA strand breaks in rat liver cells were evaluated using the comet assay. The parameters, including head DNA%, tail DNA%, and tail length, are presented in Table 1. Rats administered paracetamol at doses of 500 mg/kg and 1000 mg/kg (Group II and Group IV, respectively), exhibited a significant increase in tail DNA% and tail length, while head DNA% showed a significant decrease compared to the control group (Group I). However, co-treatment with chitosan nanoparticles (Group V and Group VI) mitigated the changes observed in the liver tissues, as reflected in the reduction of these parameters compared to those observed in cells treated with paracetamol alone (Figure 5).

Parame-ter	Group I	Group II	Group III	Group IV	Group V	Group VI
Head DNA%	99.25 ± 1.04	100.07 ± 2.94	89.20±1.82*	55.86±1.16*	98.64±2.17	96.95±3.02
Tail DNA%	0.74 ± 1.06	0.07±2.59	10.79±1.40*	45.13±1.56*	1.34±2.16	3.055±1.12*
Tail Length	5.01 ± 1.98	3.28±2.08	15.04±1.08*	58.63±0.97*	7.33±3.17	9.461±2.18*

Table 1: Comet assay in rats treated with paracetamol only or combination with chitosan nanoparticles.
Group I: control group; Group II: rats received chitosan nanoparticles at a concentration of 15 mg/kg; Group III: rats received paracetamol at a concentration of 500 mg/kg; Group IV: rats received paracetamol at a concentration of 1000 mg/kg; Group V: rats received a combination of paracetamol (500 mg/kg) and chitosan nanoparticles (15 mg/kg); Group VI: rats received a combination of paracetamol (1000 mg/kg) and chitosan nanoparticles (15 mg/kg);The values are presented as mean ± standard error (n = 10 animals in each group); *: Significant difference at $p \leq 0.05$ compared to Group I.

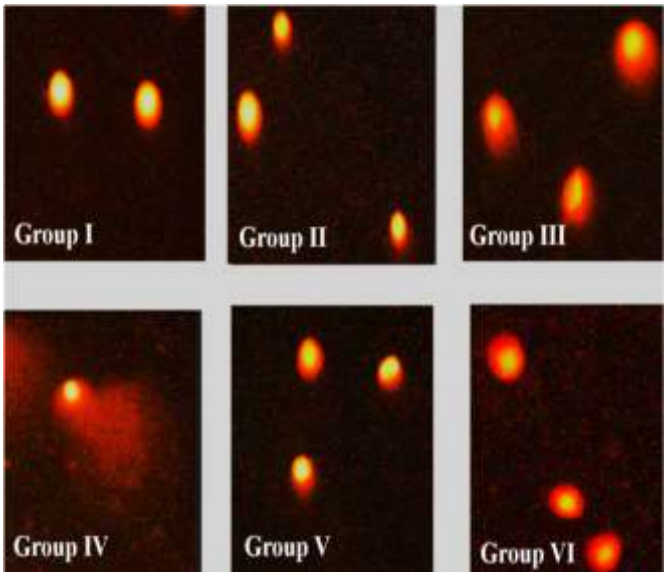

Figure 5: Representative images of the liver cells assayed by the comet assay. Group I: control group; Group II: rats received chitosan nanoparticles at a concentration of 15 mg/kg; Group III: rats received paracetamol at a concentration of 500 mg/kg; Group IV: rats received paracetamol at a concentration of 1000 mg/kg; Group V: rats received a combination of paracetamol (500 mg/kg) and chitosan nanoparticles (15 mg/kg); Group VI: rats received a combination of paracetamol (1000 mg/kg) and chitosan nanoparticles (15 mg/kg).

The comet assay revealed significant damage upon paracetamol treatment at doses of 500 mg/kg and 1000 mg/kg. The elevated tail DNA% and tail length, along with a reduced head DNA%, indicated a notable genotoxic effect of paracetamol, which aligns with previous

report.[20] The observed increase in tail DNA% in rat liver cells is genetically linked to the covalent binding of NAPQI to mitochondrial proteins. This binding disrupts mitochondrial integrity, causing membrane permeabilization and lysis. Consequently, endonucleases are released from the mitochondria, translocate to the nucleus, and induce nuclear DNA fragmentation.[27] Conversely, treatment with chitosan nanoparticles reduced both tail DNA% and tail length. The high free radical scavenging activity of chitosan nanoparticles plays a significant role in reducing DNA injury.[28] Because chitosan nanoparticles have a high flavonoid and polyphenol content, they can inhibit DNA damage. Chitosan nanoparticles decrease DNA fragmentation, consistent with previous findings.[29]

Conclusion

This study demonstrated the protective potential of chitosan nanoparticles against paracetamol-induced liver damage in white male albino rats. Paracetamol at both therapeutic and toxic doses caused significant histopathological and genetic damage, including liver fibrosis, necrosis, and DNA strand breaks. Co-administration with chitosan nanoparticles preserved liver structure and minimized cellular and genetic damage. These findings highlight chitosan nanoparticles as a promising therapeutic agent for mitigating drug-induced hepatotoxicity, with potential applications in nanomedicine and drug delivery systems.

Conflict of Interest

The author`s declare no conflict of interest.

Acknowledgments

The authors would like to express their gratitude to the College of Medicine, University of Baghdad (https://uobaghdad.edu.iq), for their support.

Funding

The author is grateful to Professor Chungui Lu and Nottingham Trent University for their support.

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