

RESEARCH ARTICLE

Nanoengineered Metformin Delivery System Targeting the AMPK/PI3K/Akt/mTOR Pathway: A Potential Therapeutic Strategy Against Cisplatin-Induced Nephrotoxicity

Shereen H. Noshi¹, Sherine M. Ibrahim^{2,*}, Omnia F. Hassan³ and Mervat Shafik Ibrahim¹

¹Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza, Egypt; ²Biochemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza, Egypt; ³Pharmacology Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza, Egypt

Abstract: Background: Cisplatin, an effective antitumor drug, is clinically limited by its dose-dependent nephrotoxicity. Rational nanocarrier design offers a strategy to improve drug delivery and minimize off-target effects. In this work, we developed a metformin-loaded mesoporous silica nanoparticle (MSN) system, which was further coated with chitosan (CTS) and tailored into distinct sizes and geometries (elliptical vs. rounded) to enhance bioactivity, kidney targeting, and biocompatibility.

Methods: Thirty-five rats were divided into seven groups after nephrotoxicity induction with cisplatin (2 mg/kg, intraperitoneally, once weekly for 4 weeks). Groups were treated orally with metformin, uncoated MET-MSNs, or CTS-coated MET-MSNs (elliptical and rounded; 40 mg/kg orally daily for 4 weeks). Biochemical markers, histology, and immunohistochemistry were performed to evaluate renal function and tissue remodeling.

Results: Cisplatin exposure induced oxidative stress, elevated TGF- β 1, TNF- α , IL-1 β , and caspase-3 levels, and caused renal dysfunction with marked histopathological damage. Both MET and CTS-MET-MSNs mitigated these changes, with geometry- and coating-dependent improvements in renal biomarkers and tissue recovery. qPCR analysis showed that CTS-MET-MSNs significantly downregulated cisplatin-induced AMPK/PI3K/Akt and restored mTOR expression. CTS-coated carriers showed superior anti-inflammatory, antioxidant, and anti-apoptotic effects compared with free MET.

Discussion: In light of the available evidence, the design of our carrier system was guided by key parameters known to enhance kidney targeting. Rounded nanoparticles were selected since their geometry has been reported to promote efficient circulation, facilitate passage through the glomerular filtration barrier, and improve interaction with renal tissues. In addition, surface functionalization with chitosan was introduced to confer a positive charge, which is expected to favor electrostatic interactions with the negatively charged renal cell membranes. Accordingly, for our formulation strategy, rounded particles with a CH coating were deliberately chosen to maximize kidney selectivity. The morphological assessment of CTS-MET-MSNs by TEM showed the successful formulation of the rounded nanoparticles with a defined mesoporous structure.

Conclusion: This study demonstrated that the CTS-MET-MSNs offer a superior strategy for mitigating cisplatin-induced acute kidney injury compared to free metformin. By optimizing the vehicle's architecture using rounded geometry to promote favorable glomerular filtration kinetics and applying a positively charged chitosan coating to exploit electrostatic binding with negatively charged renal membranes. We achieved highly site-specific kidney targeting. Morphological evaluation *via* TEM confirmed the successful fabrication of these rounded nanoparticles with a well-defined mesoporous framework. Biologically, the CTS-MET-MSNs suppressed cisplatin-mediated oxidative stress, cellular damage, and histopathological degradation, outperforming free MET. Most notably, qPCR analysis revealed that this advanced nanoformulation achieved its potential anti-inflammatory, antioxidant, and anti-apoptotic properties by precisely downregulating the cisplatin-induced AMPK/PI3K/Akt pathway and restoring downstream mTOR expression. Collectively, these findings highlight CTS-MET-MSNs as a highly potent, targeted nanomedicine capable of maximizing the clinical protective window of metformin against chemotherapeutic nephrotoxicity.

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*Address correspondence to this author at the Biochemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Giza, Egypt; E-mail: sherinemahmoud@msa.eun.eg

1. INTRODUCTION

Cancer remains a leading cause of death worldwide. While chemotherapy effectively limits tumor growth, its clinical utility is often compromised by severe side effects, including neurotoxicity, infertility, and most notably nephrotoxicity [1, 2]. Cisplatin remains a cornerstone of chemotherapy for various solid malignancies, including head, neck, testicular, and ovarian cancers; however, its clinical application is severely hampered by dose-limiting nephrotoxicity, which clinically affects approximately 25% to 35% of patients following a single dose [3]. This toxic insult frequently progresses into Acute Kidney Injury (AKI), a complex syndrome that, if left unmanaged, significantly increases the risk of developing Chronic Kidney Disease (CKD) and end-stage renal failure [4]. Currently, there are no effective therapeutic agents available to fully prevent cisplatin-induced renal failure [5].

Recent research has clarified the molecular basis of cisplatin-induced damage. The clinical manifestation of cisplatin-induced AKI is characterized by a rapid decline in the glomerular filtration rate (GFR) and significant histopathological alterations that mirror human renal failure. These include severe renal tubular necrosis, predominantly in the proximal tubules, as well as interstitial fibroplasia and robust mononuclear inflammatory cell infiltration. Substantial evidence suggests that these pathologies are driven by a multifaceted “cytotoxic storm” [6]. This involves the excessive accumulation of reactive oxygen species (ROS) and the concomitant depletion of vital antioxidant enzymes, such as superoxide dismutase (SOD) and reduced glutathione (GSH), leading to irreversible oxidative damage to cellular lipids and proteins [7].

Furthermore, the disease progression is exacerbated by the activation of a pro-inflammatory cascade involving cytokines such as TNF- α and IL-1 β , which recruit immune cells to the site of injury and promote tissue remodeling. At the molecular level, recent insights highlight that the transition from acute damage to permanent structural failure is governed by the p53-mediated apoptotic pathway. This pathway interacts with the AMPK/PI3K/Akt/mTOR signaling axis, a critical metabolic and survival regulator. Dysregulation of this axis during cisplatin therapy inhibits autophagy and promotes programmed cell death in renal epithelial cells, providing a clear mechanistic link between chemotherapeutic administration and the onset of associated renal diseases [8]. These molecular mechanisms—which occur due to cisplatin treatment and include oxidative stress, inflammation, and necrosis—can play potential roles in cisplatin-induced kidney toxicity.

The accumulation of reactive oxygen species (ROS) above insufficient antioxidant molecular mechanisms leads to the reduction of glutathione (GSH) and superoxide dismutase (SOD); on the other hand, cisplatin treatment leads to the elevation of inflammatory mediators, including interleukin-1 β (IL-1 β) and Tumor Necrosis Factor-alpha (TNF) [9]. Collectively, previous studies illustrated that cisplatin-induced renal damage can be successively improved through regulation of activated protein kinase (AMPK) and its main downstream pathway, the PI3K/Akt pathway, together with the p53 molecular pathway [10].

Metformin, primarily used as a hypoglycemic drug for type 2 diabetes, has demonstrated potent antioxidant and anti-inflammatory properties [11]. Studies indicate that metformin (MET) can reduce ROS production and enhance antioxidant expression *via* the AMPK/FOXO-Trx axis [12]. While some research suggests MET may act synergistically with cisplatin, other studies report potential antagonism in certain cancer cell lines, necessitating further *in vivo* exploration to determine if MET co-administration can safely ameliorate nephrotoxicity [13, 14].

To optimize delivery, Mesoporous Silica Nanoparticles (MSNPs) have emerged as versatile platforms due to their high surface area, tunable pore size, and biocompatibility [15]. Rounded MSNPs, in particular, exhibit superior biodistribution and tissue accumulation [16]. To enhance renal targeting, MSNPs can be coated with chitosan, a biocompatible, positively charged polysaccharide [17]. A chitosan-coated system can target the negatively charged glycocalyx layer of the glomerular capsule through electrostatic interactions [18].

Based on these insights, this study aimed to develop a metformin-loaded, chitosan-coated MSNP system to mitigate cisplatin-induced nephrotoxicity in rats. The system's design utilizes two strategies for renal selectivity; Charge-Mediated Interaction: The positive zeta potential of chitosan favors interaction with the negatively charged renal glycocalyx; and Size-Dependent Filtration: The optimized hydrodynamic diameter of the nanoparticles facilitates renal accumulation *via* secretion from peritubular capillaries into the proximal tubules [19].

2. MATERIALS AND METHODS

2.1. Materials

Metformin hydrochloride was gifted by EVA Pharm (Egypt). Mesoporous silica (50 nm rounded and rounded) was purchased from Nanotech (Egypt). Chi-

tosan (medium molecular weight) and glacial acetic acid were procured from Sigma-Aldrich (USA). Sodium phosphate dibasic, potassium chloride, and potassium dihydrogen phosphate were obtained from Al Nasr Pharmaceuticals Chemical Company (Egypt). Cisplatin (cisPtCl₂ (NH₃)₂) was supplied by Oncotec Pharma Company. All other chemicals employed were of pharmaceutical grade.

2.2. Loading Metformin (MET) on Mesoporous Silica Nanoparticles (MSNPs)

Metformin was loaded onto mesoporous silica nanoparticles (MSNPs) by dispersing 0.1 g of MSNPs in 10 mL of a 400 ppm aqueous metformin solution (pH 7) [20]. The suspension was continuously agitated for 24 h to facilitate drug adsorption onto the nanoparticle surface and within the mesopores. The mixture was then subjected to vacuum filtration to remove unbound metformin, and the resulting metformin-loaded nanoparticles (MET-MSNPs) were collected for further use [21].

2.3. Coating Metformin-loaded Mesoporous Silica Nanoparticles (MET-MSNPs) with Medium Molecular Weight Chitosan

A 0.6% (w/v) chitosan solution was prepared in 10% (v/v) acetic acid and adjusted to pH 6. MET-MSNPs were dispersed in this solution and stirred for 48 h to facilitate surface coating. Excess chitosan was removed by ultracentrifugation, and the chitosan-coated nanoparticles (CTS-MET-MSNPs) were collected, freeze-dried, and stored in tightly sealed containers under dry conditions until further use [22].

2.4. Characterization of CTS-MET-MSNPs

2.4.1. Drug Entrapment Efficiency

The entrapment efficiency (EE%) of CTS-MET-MSNPs was determined indirectly by measuring the amount of free drug present in the supernatants obtained during the preparation steps. After the loading process, the dispersion was centrifuged at 15,000 rpm for 30 min at 4 °C, and the supernatant was collected. The MET-MSNPs nanocarrier was washed once with the same medium, centrifuged under the same conditions, and the wash supernatant was also collected. Following the coating step, centrifugation and washing were repeated, and the supernatants were combined. The concentration of untrapped metformin (D_u) in the pooled supernatants was quantified spectrophotometrically at $\lambda_{\max}$ 234 nm using a Shimadzu UV-2401/PC spectrophotometer. The total drug amount (D) corresponded to the amount of metformin

initially added during the loading process. EE% and LC% were calculated according to the following equations [23]:

$$EE(\%) = \frac{Dt \text{ (total amount of MET)} - Du \text{ (free untrapped MET)}}{Dt \text{ (total amount of MET)}} \times 100$$

$$LC(\%) = \frac{\text{Weight of MET in nanoparticles}}{\text{total weight of nanoparticles}} \times 100$$

Where Dt represents the total drug amount and Du the untrapped drug amount quantified in the pooled supernatants.

2.4.2. Particle Size (PS), Polydispersity Index (PDI) and Zeta Potential (ZP)

The particle size (PS) and polydispersity index (PDI) of the prepared nanoparticles were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Malvern Instruments, UK) at 25 °C. Samples were diluted with distilled water to obtain an appropriate scattering intensity before measurement. The PDI, which reflects the width of the size distribution, was automatically calculated by the instrument. A lower PDI value indicates a more homogeneous particle size distribution. All measurements were carried out in triplicate, and results are presented as mean $\pm$ SD.

The zeta potential (ZP) was determined on the same instrument by electrophoretic mobility measurements in double-distilled water. Each sample was analyzed in triplicate, and the results were expressed as mean $\pm$ SD. A ZP value greater than ± 30 mV was considered indicative of good colloidal stability [24].

2.4.3. In Vitro Drug Release

Drug release was evaluated using the membrane diffusion method for MET aqueous solution and CTS-MET-MSNPs. A cellulose membrane, pre-soaked in phosphate-buffered saline (PBS, pH 7.4) for 24 h, was loaded with 1 mL of each sample (equivalent to 0.4 mg/mL MET). The membrane was then immersed in 100 mL PBS (pH 7.4) and incubated at 37 ± 0.5 °C under shaking at 50 rpm. At predetermined intervals, 5 mL aliquots were withdrawn and immediately replaced with fresh PBS to maintain sink conditions. The amount of released MET was determined spectrophotometrically at $\lambda_{\max}$ 234 nm. Results are expressed as mean $\pm$ SD of three independent experiments [25].

2.4.4. Transmission Electron Microscope (TEM)

The morphology of MSNPs and CTS-MET-MSNPs was examined using transmission electron microscopy (TEM, JEM-2100, JEOL, Japan) operated at an acceleration voltage of 80 kV. Samples were prepared by diluting nanoparticle dispersion in ethanol, placing one drop on a carbon-coated copper grid, and allowing it to air-dry. The dried samples were negatively stained with 2% (w/v) phosphotungstic acid, dried again, and then imaged under TEM.

2.4.5. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were recorded for chitosan, MET, MSNPs, MET-MSNPs, and CTS-MET-MSNPs. For each analysis, 2 mg of sample was mixed with dry KBr and compressed into a pellet. Spectra were obtained using a Shimadzu IR Affinity spectrometer (Shimadzu, Japan) over the range 400-4000 cm^{-1} [26].

2.5. Animal Experimentation

Thirty-five Wistar male rats (approximately 110-120 g in weight) were purchased from the Nile Pharmaceutical Company, Cairo, Egypt. All rats were allowed to adapt in cages for a week before starting the experiment. Rats with evidence of renal dysfunction, systemic disease, infection, or physical abnormalities before treatment initiation were excluded from this study. Rats were kept in cages at a temperature of $25 \pm 2^\circ\text{C}$ with a light and dark cycle. Animals were randomly allocated to experimental groups using a simple randomization method based on a computer-generated random number table, ensuring equal group sizes. The rats in cages were accommodated with free access to water and food.

All animal experiments were carried out according to the guidelines of the ethical committee in the Faculty of Pharmacy at MSA University (ethical code: PB37/REC37/2024P). This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices. The use of male Wistar rats, a well-established model for cisplatin-induced nephrotoxicity, supports internal validity and allows comparison with a substantial body of pre-clinical literature. Key pathological features observed in this model, including oxidative stress, inflammation, and tubular injury, closely resemble those reported in human cisplatin-associated acute kidney injury, suggesting biological relevance.

2.6. Experimental Design

Thirty-five male Wistar rats were randomly divided into seven equal groups ($n = 5$ per group) and housed in separate cages using a computer-generated random sequence to ensure unbiased sample distribution. The sample size ($n=5$ per group, total $N=35$) was determined a priori using G*Power software (version 3.1.9.7). A one-way fixed effects ANOVA was used with an effect size $f = 0.95$, $\alpha = 0.05$, and power ($1-\beta$) = 0.95. This effect size was estimated based on preliminary data showing a projected 40% difference in serum creatinine between the Cis-intoxicated and MET-treated groups. The groups were assigned as follows: *Group 1*: served as the control group (control); it was used to establish baseline physiological parameters for comparison with cisplatin-induced pathology and received distilled water orally and was injected with normal saline once a week for 4 weeks intraperitoneally (i.p). For treatment groups, as shown in Fig. (1a), treatment started on the day of the first cisplatin injection *Group 2*: Cisplatin-intoxicated group (Cis) rats were injected with 2 mg/kg of cisplatin weekly for 4 weeks intraperitoneally. The cisplatin dosage regimen (2 mg/kg weekly, cumulative 8 mg/kg) was selected to induce a model of chronic-like progressive renal injury rather than a single-dose acute insult, allowing for the evaluation of the long-term protective effects of the nano-formulations. *Group 3*: intoxicated group treated with metformin; treatment started on the day of the first cisplatin injection (Cis + MET) with a dose of 200 mg/kg MET by oral gavage daily for 28 days and injected with 2 mg/kg of CIS i.p weekly for 4 weeks. *Group 4*: intoxicated group treated with elliptical metformin MSNPs (Cis + MET-MSNPs-elliptical), Rats received MET-loaded MSNPs delivering 40 mg MET/kg/day (corresponding to 200 mg NP/kg/day; LC = 20% due to morphological variations in entrapment efficiency, initial drug-to-carrier feeding ratios were adjusted to yield a uniform final loading capacity of 20% across all formulations.) by oral gavage for 4 weeks, the 40 mg/kg dose in the nano-formulation was chosen to demonstrate the dose-sparing effect (enhanced potency at lower concentrations) compared to the standard 200 mg/kg dose of free MET, The 40 mg/kg nano-formulation dose represents a 5-fold reduction ($1/5^{\text{th}}$) of the standard free MET dose (200 mg/kg), This dose-sparing strategy was selected based on established literature demonstrating that polymeric and silica-based nanocarriers significantly enhance the gastrointestinal retention, sustained release, and oral bioavailability of metformin, thereby achieving equivalent or superior pharmacodynamic outcomes at reduced active concentrations [27, 28]. This served as a direct test of the

nanocarriers' ability to enhance bioavailability and therapeutic index, in addition to cisplatin (2 mg/kg, i.p., once weekly for 4 weeks "The nanoparticle dose was calculated according to the equation: "Administered NP dose (mg/kg) = MET-equivalent dose (mg/kg) ÷ LC. Thus, $40 \div 0.20 = 200$ mg NP/kg/day". *Group 5*: intoxicated group treated with rounded metformin MSNPs (Cis + MET-MSNPs-rounded), Rats received MET-loaded MSNPs delivering 40 mg MET/kg/day (corresponding to 200 mg NP/kg/day; LC = 20%) by oral gavage for 4 weeks, in addition to cisplatin (2 mg/kg, i.p., once weekly for 4 weeks). *Group 6*: intoxicated group treated with elliptical CTS-MET-MSNPs (Cis + CTS-MET-MSNPs-elliptical), Rats received MET-loaded MSNPs delivering 40 mg MET/kg/day (corresponding to 200 mg NP/kg/day; LC = 20%) by oral gavage for 4 weeks, in addition to cisplatin (2 mg/kg, i.p., once weekly for 4 weeks). *Group 7*: intoxicated group treated with rounded CTS-MET-MSNPs (Cis + CTS-MET-MSNPs-rounded). Rats received MET-loaded MSNPs delivering 40 mg MET/kg/day (corresponding to 200 mg NP/kg/day; LC = 20%) by oral gavage for 4 weeks, in addition to cisplatin (2 mg/kg, i.p., once weekly for 4 weeks). Intraperitoneal injections and oral gavage were carried out using appropriate techniques and volumes to reduce discomfort. By the end of the experiment, rats were anesthetized with isoflurane to collect blood samples from rats. All samples were then centrifuged at 3000 r/min for 20 min. Then, rats were euthanized by cervical dislocation under deep anesthesia, in accordance with accepted humane practices. All kidneys were harvested from all groups for biochemical, histopathological, and

immunohistochemical analyses. To ensure unbiased analysis, histopathological and immunohistochemical assessments were performed by a pathologist who was blinded to the experimental group assignments. No unexpected adverse events, procedure-related injuries, or treatment-related mortalities occurred during the study. No adverse reactions attributable to metformin or nanoparticle formulations were observed. Predefined humane endpoints were established before study initiation. Animals were monitored for clinical signs of distress, including excessive body weight loss (>20%), persistent lethargy, abnormal posture, reduced mobility, impaired grooming, dehydration, and reduced food or water intake. Monitoring was performed daily, with body weight recorded weekly. Animals reaching humane endpoint criteria were to be immediately removed from the study and humanely euthanized to prevent unnecessary suffering. For molecular quality control, isolated RNA purity was verified *via* spectrophotometry (A260/280 ratio: 1.8-2.0), and RT-PCR was performed in triplicate with internal normalization. Furthermore, to ensure the quality of histopathological and immunohistochemical data, analysis was performed by a blinded pathologist to prevent observer-related bias.

2.7. Biochemical Analysis

2.7.1. Assessment of Renal Function Tests

Urea and creatinine levels were assayed in serum using specific diagnostic kits (Biodiagnostics, Giza, Egypt).

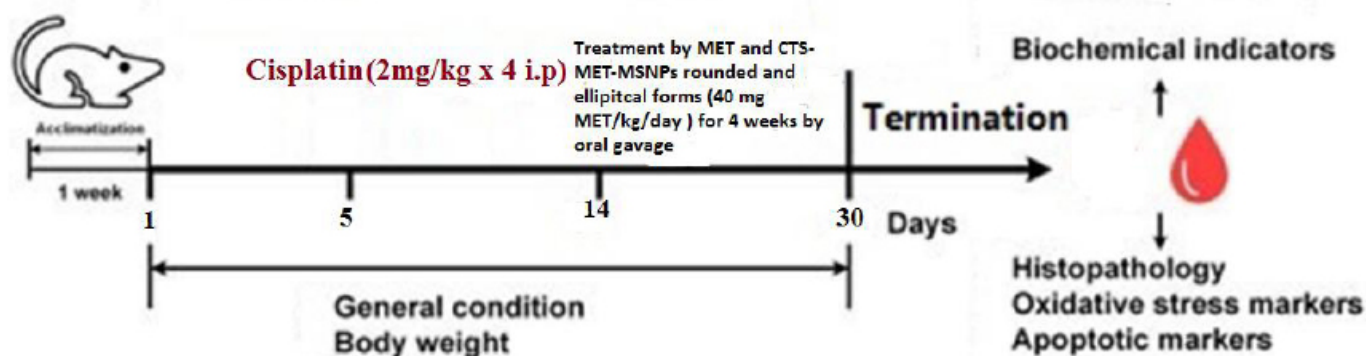


Fig. (1a). Schematic of the experimental timeline. Rats were acclimatized for 7 days. Starting at Day 0, Cisplatin (2 mg/kg) was administered intraperitoneally (i.p.) once weekly for 4 weeks. Treatment groups received daily oral gavage of MET or MET-nanoparticles simultaneously for 28 days. Terminal euthanasia and sample collection occurred on Day 29. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

2.7.2. Assessment of Oxidative Stress Markers

Lipid peroxidation products were measured by the thiobarbituric acid (TBA) test to determine malondialdehyde (MDA) levels in renal tissue homogenates. In addition, reduced glutathione (GSH) was assayed using the dithio-binitrobenzoic acid method. Finally, superoxide dismutase (SOD) activity was determined in renal homogenates using a commercial kit (Biodiagnostics, Egypt).

2.7.3. Assessment of Inflammatory and Apoptotic Markers

Interleukin-1 β (IL-1 β), transforming growth factor- β 1 (TGF- β 1), and tumor necrosis factor- α (TNF- α) were quantified in renal tissue homogenates using enzyme-linked immunosorbent assay (ELISA) kits (MyBiosource, Inc., San Diego, USA) according to the manufacturer's instructions. Additionally, apoptotic markers, including caspase-3 and kidney injury molecule-1 (KIM-1), were determined by ELISA (BioSource Int., San Diego, USA).

2.7.4. Assessment of AMPK, PI3K, Akt, and mTOR Gene Expression in Renal Tissue Homogenate via Quantitative Real-time Polymerase Chain Reaction (qPCR)

To estimate the changes in mRNA gene expression for AMPK, PI3K, Akt, and mTOR, TRIzol reagent (Life Technologies, USA) was used to isolate the total RNA from 60 mg of renal tissue. Consequently, the reverse transcriptase (Invitrogen) enzyme was used to make the first strand of complementary DNA (cDNA) using a 1 mg RNA template. The RT-PCR was performed in a StepOnePlus thermal cycler (Applied Biosystems, Waltham, MA, USA) using the Sequence Detection Software [29]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. First, the C_t values of the target genes (AMPK, PI3K, Akt, mTOR) were normalized to the C_t of the internal control (beta-actin) to obtain ΔC_t . Then, the ΔC_t of the treatment groups was compared to the ΔC_t of the control group to calculate ΔC_t . All collected samples were allowed to run in triplicate, and cDNA levels were normalized to β -Actin using specific primers (Table 1).

2.8. Histopathological Assessment of Renal Tissue

A histological assessment was performed on kidney tissues from all studied groups to clarify and investigate the histological changes that were induced by cisplatin and other treatments. The kidney tissues of all studied groups were first fixed in 10% formalin,

embedded in paraffin, then sliced into sections at a thickness of 5 μ m, and finally stained with H&E [30]. Briefly, the determination of the severity of renal damage was assessed according to the presence of these criteria: degeneration of the tubular epithelium and glomeruli, infiltration and necrosis of the glomeruli and tubular cells, and interstitial renal fibrosis.

Table 1. Primers used in RT-PCR.

AMPK	F: 5'-TGATGACCATGTGCCAAC-3'
	R: 5'-CCCTGATATCTTTGATTGTGG-3'
PI3K	F: 5'-AGATGAGTGACCGAGGAGGT-3'
	R: 5'-CAAAACCAGCCAGAGGCACT-3'
Akt	F: 5'-CTCTTTCCAGACCCACGACC-3'
	R: 5'-ACAGGTGGAAGAACAGCTCG-3'
mTOR	F: 5'-CTTGCTGAAGTGGAGGCTGATGG-3'
	R: 5'-CCGTTTTCTTATGGGCTGGCTCTC-3'
β -Actin	F: 5'-GGTGAAGGTGACAGCAGT-3'
	R: 5'-TGGGGTGGCTTTTAGGAT-3'

2.9. Immunohistochemistry Assessment of P 53 in Renal Tissue

Briefly, renal tissues were sliced into sections of 5 μ m thickness and exposed to deparaffinization, followed by a heat-induced antigen retrieval process and the blocking of endogenous peroxidase. After washing, the renal tissues were then incubated with primary antibodies (1:100 anti-P53, Santa Cruz Biotechnology Inc.) in PBS overnight. Subsequently, HRP-labelled secondary antibodies (Abcam, UK) were applied for 30 min after another wash. A DAB substrate kit was finally used for the development of color, and counterstaining with Mayer's hematoxylin was applied [31].

2.10. Statistical Analysis

Sample size was determined a priori using G*Power software (version 3.1.9.7) for a one-way fixed effects ANOVA. The calculation was based on a large effect size $f = 0.95$ (derived from preliminary pilot data which showed significant separation between MET-M-SNPs and control groups), $\alpha = 0.05$, and power $(1 - \beta) = 0.95$, yielding a total $N = 35$ ($n = 5$ per group). All data are expressed as mean $\pm$ SEM. The normality of data distribution was verified using the Shapiro-Wilk test. Differences between the control group and the six experimental treatment groups were analyzed using one-way analysis of variance (ANOVA). To identify specific inter-group differences (e.g., MET-M-SNPs vs. free MET), Tukey's post-hoc test was employed; this test was selected to strictly control the fam-

ily-wise error rate across the multiple pairwise comparisons. Statistical significance was set at $P < 0.05$, with higher levels of significance denoted as $P < 0.01$ and $P < 0.001$ where applicable. All calculations were performed using GraphPad Prism software version 6.0 (La Jolla, CA, USA)."

3. RESULTS

3.1. Characterization of Metformin-loaded Mesoporous Silica Coated with Chitosan (CTS-MET-MS-NPs)

The physicochemical properties of the prepared nanoparticles are summarized in Table 2. Both elliptical and rounded bare MSNPs exhibited mean particle sizes around 50 nm with low PDI values (<0.25), reflecting a uniform size distribution. Upon metformin loading, particle size increased slightly for both shapes (49.7 ± 1.2 to 51.2 ± 1.5 nm for elliptical; 52.7 ± 1.3 to 90.1 ± 4.5 nm for rounded). The increase was more pronounced in rounded MSNPs, suggesting shape-related differences in drug-carrier interaction.

Chitosan coating markedly increased particle size in both shapes, with CTS-MET-MSNPs-elliptical reaching 180.4 ± 3.6 nm and CTS-MET-MSNPs-rounded 103 ± 2.6 nm. The coating also increased PDI values (>0.3), consistent with broader size distributions typically associated with polymer-modified nanocarriers. Free MSNPs showed negative zeta potentials of approximately -19 mV, related to their surface silanol groups. Metformin loading caused partial neutralization of this charge (-15.0 mV and -9.0 mV for elliptical and rounded, respectively), which is consistent with the electrostatic interactions between the anionic silica surface and cationic metformin. The chitosan coating also shifted the zeta potential to positive values (+10.0 mV for elliptical; +34.0 mV for rounded), proving the successful colloidal stability and surface modification.

Entrapment efficiency (EE) changed significantly between the two geometries: MET-MSNPs-rounded nanoparticles achieved ~43% EE, which was approximately four-fold higher than that of MET-MSNPs-elliptical nanoparticles (~10%).

3.2. Structural Analysis of CTS-MET-MSNPs

3.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopic analysis was carried out to investigate the chemical composition of the nanocarriers and confirm drug-polymer-silica interactions. The spectrum of bare MSNPs showed the typical silica framework peaks: a broad band around 3460 cm^{-1} (O-H stretching), a strong band at 1110 cm^{-1} (Si-O stretching), and a peak at 815 cm^{-1} (Si-O symmetric stretching) (Fig. 1b). Chitosan displayed characteristic absorption bands at 3596 cm^{-1} , 3420 cm^{-1} , 1655 cm^{-1} , 1589 cm^{-1} , 1380 cm^{-1} , and 1150 cm^{-1} , corresponding to hydroxyl, amine, amide, and methyl functional groups (Fig. 1b) [32, 33]. Metformin exhibited distinctive peaks at 3445.98 cm^{-1} (N-H stretching), 2080.31 cm^{-1} (C=N stretching), 1632.69 cm^{-1} (C=O stretching), 1113.23 cm^{-1} (C-N stretching), and 619.60 cm^{-1} (N-H bending), consistent with its reported structure (Fig. 1b) [32].

The spectrum of CTS-MET-MSNPs showed the co-existence of these characteristic peaks, confirming the successful incorporation of both chitosan and metformin into the silica matrix [33]. Importantly, broadening and slight shifts of silica-related bands, particularly the Si-O stretching vibration, suggested possible hydrogen bonding and electrostatic interactions between the silica surface and the incorporated molecules (Fig. 1b). These spectral shifts support the successful coating of MSNPs and are consistent with earlier reports of CH of nanoparticle systems [34, 35].

Table 2. Physicochemical properties of prepared nanoparticles. Data points represent mean values $\pm$ standard deviation (SD) of triplicate measurements (n=3).

	Particle Size (nm) $\pm$ SD	PDI $\pm$ SD	Zeta Potential (ZP) (mV) $\pm$ SD	Entrapment Efficiency (EE%) $\pm$ SD
MSNP-elliptical	49.7 ± 1.2	0.24 ± 0.03	-19.2 ± 0.8	NA
MET-MSNP-elliptical	51.2 ± 1.5	0.32 ± 0.04	-15.0 ± 0.9	10.42 ± 0.52
CTS- MET-MSNP-elliptical	180.4 ± 3.6	0.53 ± 0.06	10.0 ± 1.0	10.73 ± 0.54
MSNP-Rounded	52.7 ± 1.3	0.19 ± 0.02	-19.7 ± 0.7	NA
MET-MSNP-Rounded	90.17 ± 4.5	0.43 ± 0.05	-9.0 ± 0.9	43.21 ± 2.16
CTS- MET-MSNP-Rounded	103 ± 2.6	0.32 ± 0.04	34.0 ± 1.7	42.69 ± 2.13

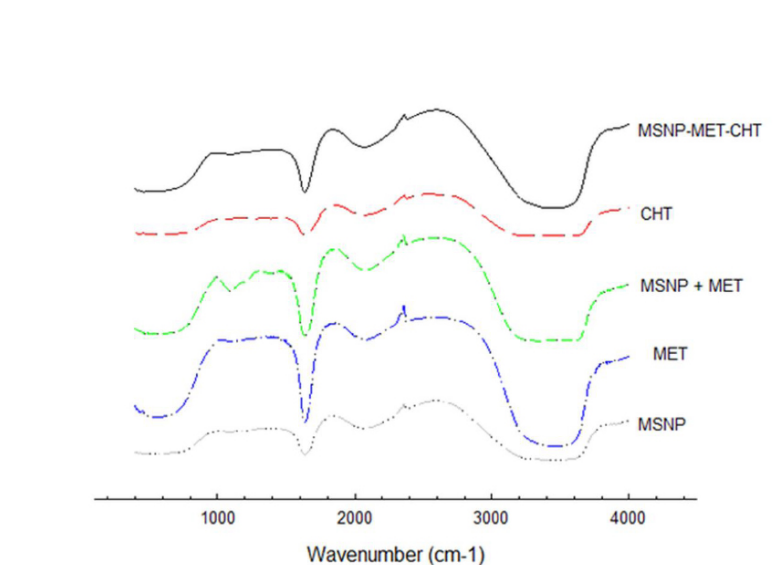


Fig. (1b). Spectra were recorded over the range 400–4000 cm^{-1} using the KBr pellet method to confirm chemical composition and drug-polymer-silica interactions. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.2.2. Transmission Electron Microscopy (TEM)

TEM analysis showed that rounded MSNPs with an average size of ~ 50 nm, were consistent with the measurements of DLS (Fig. 2A). The defined porous channels of the silica matrix lead to their mesoporous na-

ture that is considered essential for enhancement of drug loading owing to their pore volume and high surface area [36]. After chitosan coating, nanoparticles exhibited a rounded morphology but showed an average larger diameter of ~ 110 nm (Fig. 2B), as confirmed also by the DLS measurements.

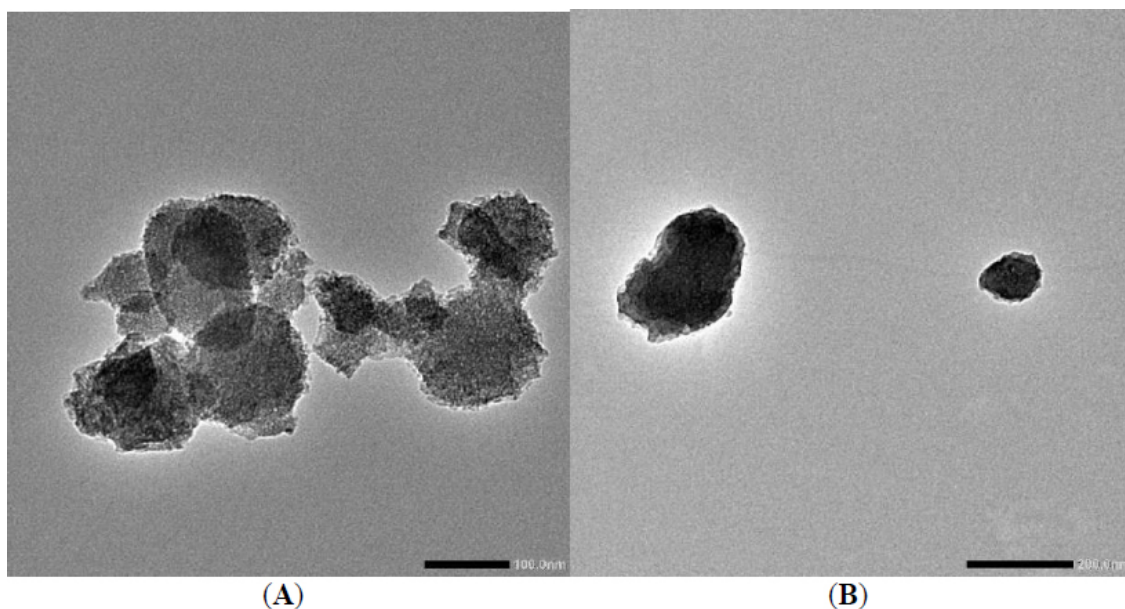


Fig. (2). (A) Bare rounded MSNPs showing a mesoporous framework (~ 50 nm). (B) CTS-coated MSNPs exhibiting an increased diameter (~ 110 nm). Imaging was performed at an acceleration voltage of 80 kV using negative staining with 2% phosphotungstic acid. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.3. *in vitro* Release Behavior of CTS-MET-MSN Nanoparticles

Previous studies on drug release were done at pH 7.4 to mimic physiological conditions. Free MET solution showed a significant release, with about 100% release finished within 30 minutes (Fig. 3). On the other hand, MET-MSNs revealed a better sustained release, with about ~80% of MET released after 4 h, showing the gradual diffusion of drug molecules *via* the mesoporous silica nanoparticles. The CTS-MET-MSNs formulation exhibited the best prolonged release profile, releasing only about ~84% of the drug over 24 h, reflecting the essential influence of the chitosan coating on drug diffusion retarding.

Kinetic modeling also supported these results, with the CTS-MET-MSN results best fitting the Higuchi model ($R^2 = 0.998$), which indicated diffusion-controlled release. To match the acidic lysosomal environment that is (pH $\approx$ 5-6) that occurs after kidney uptake, the release was also detected at pH 5.5 (Fig. 3). Interestingly, while free metformin exhibited comparable release at both pH values, MET-MSNs demonstrated a faster release under acidic conditions (~70% in 2 h). Likewise, CTS-MET-MSNs released metformin more rapidly at pH 5.5 than at pH 7.4, though still in a sustained manner. The release kinetics again followed the Higuchi model ($R^2 = 0.997$), confirming diffusion as the dominant release mechanism even under acidic conditions [37].

3.4. Biochemical Analysis

3.4.1. MET and CTS-MET-MSN Improved Kidney Function Tests

To assay kidney dysfunction that is induced by Cis, in our study the serum levels of urea and creatinine were measured in rats treated with MET, MET-MSNs rounded and elliptical nanoparticles and CTS-MET-MSNs rounded and elliptical nanoparticles. As illustrated in Fig. (4), the induction of rats by Cis caused a significant increase in both serum urea (58.4 ± 1.5 mg/dl) and creatinine (32.6 ± 2.1 mg/dl) compared to the control group (44.5 ± 0.8 mg/dl and 8.6 ± 0.9 mg/dl, respectively). Administration of MET with Cis attenuated serum urea (52.8 ± 1.9 mg/dl) and creatinine (18.8 ± 8.4 mg/dl) significantly ($P < 0.05$). Additionally, the CIS + MET-MSNs group significantly reduced serum creatinine levels ($P < 0.05$) compared to the cisplatin group, with rounded and elliptical forms reaching 18.2 ± 0.6 mg/dl and 21.7 ± 1.8 mg/dl, respectively. Rats treated with either the rounded or elliptical CTS-MET-MSNs exhibited a substantial alteration in kidney function tests, as evidenced by a more pronounced reduction in serum creatinine ($P < 0.05$), specifically reaching 13.4 ± 1.7 mg/dl for the rounded CTS-MET-MSNs, which represents a nearly 60% reduction in creatinine compared to the Cis group, consistent with the pH-responsive release and protective performance of the CTS-modified nanocarrier system.

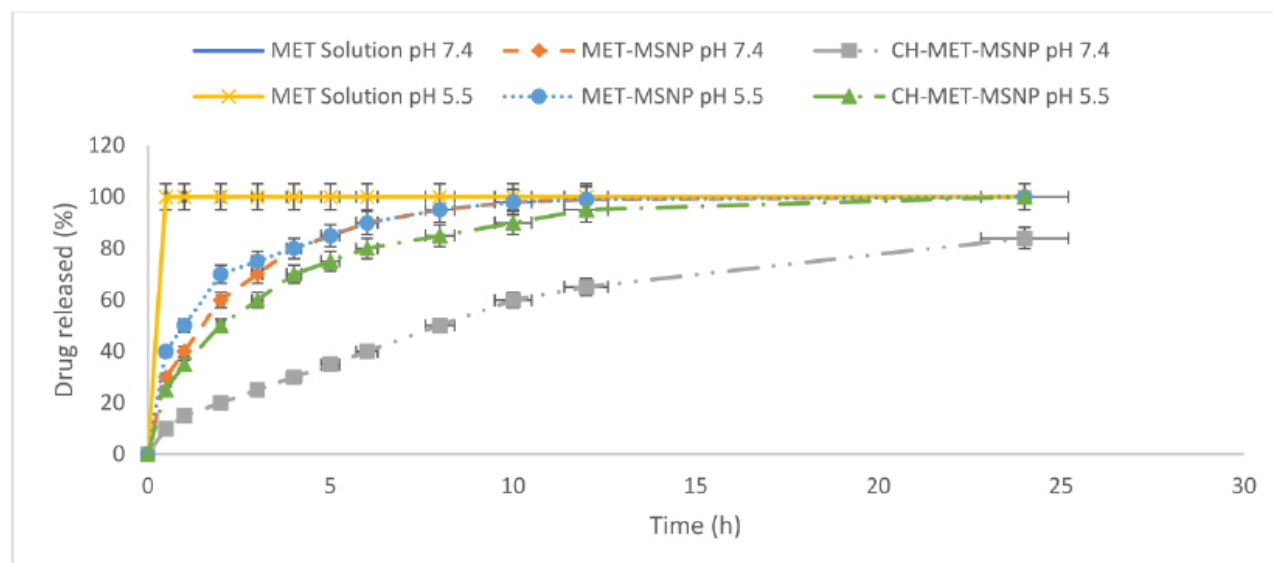


Fig. (3). Release was monitored at pH 7.4 (physiological) and pH 5.5 (acidic/lysosomal) using the membrane diffusion method. Data are expressed as mean $\pm$ SD of three independent experiments ($n=3$). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

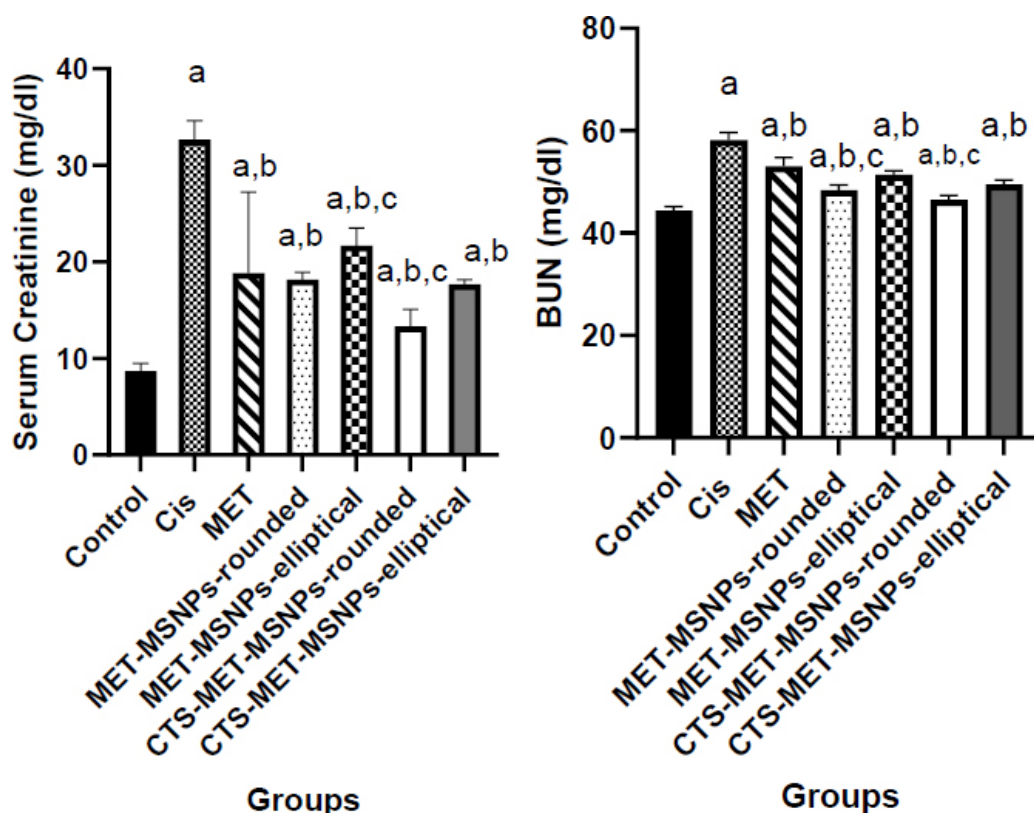


Fig. (4). Serum levels of Urea and Creatinine were measured *via* diagnostic kits. Data are presented as mean $\pm$ SEM (n=5). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test: ^a $P < 0.05$ vs. control group, ^b $P < 0.05$ vs. cisplatin (Cis) group, ^c $P < 0.05$ vs. free MET-treated group. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.4.2. MET and CTS-MET-MSN Counteract Cis-Induced Renal Oxidative Stress

Administration of cisplatin exhibited prominent oxidative stress in kidney tissues, which is indicated by a considerable increase in MDA levels (71.2 ± 1.8 $\mu\text{mol/g}$ tissue) ($P < 0.05$) and a significant suppression in SOD (43.8 ± 1.9 U/mg protein) and GSH activity (25.4 ± 2.3 mmol/mg tissue) ($P < 0.05$) relative to the control group (38.5 ± 1.3 $\mu\text{mol/g}$, 81.6 ± 1.9 U/mg, and 74.5 ± 3.1 mmol/mg, respectively), indicating the presence of oxidative damage. On the contrary, administration of MET to Cis-treated rats did not significantly restore the renal MDA (69.2 ± 1.2 $\mu\text{mol/g}$) and SOD levels (43.8 ± 0.6 U/mg) ($P < 0.05$). Regarding SOD levels ($P < 0.05$), the Cis + MET-MSNPs rounded groups revealed a significant elevation in renal tissue (57.4 ± 1.5 U/mg) when compared to the Cis group; while administration with Cis and CTS-MET-MSNPs rounded nanoparticles exhibited a potential antioxidant activity in kidney tissues (SOD: 66.4 ± 1.6 U/mg; GSH: 64.2 ± 0.9 mmol/mg) compared to the Cis + MET-MSNPs

and Cis groups (Fig. 5). Notably, the CTS-MET-MSNPs formulations (rounded and elliptical) exhibited superior antioxidant effects, which may be attributed to the structural design and surface functionalization of the nanocarrier that were previously optimized for stability, controlled release, and enhanced potential for renal tissue interaction. This enhanced effect is consistent with the reported anti-inflammatory and antioxidant activities of both chitosan and metformin, suggesting a possible synergistic contribution from the drug-polymer combination. Furthermore, the selection of a 40 mg/kg dose for the nano-formulations (representing a 5-fold reduction compared to free MET) proved that nanoengineered delivery systems can significantly enhance the therapeutic index of drugs. For instance, chitosan-coated MSNPs have been shown to improve bioavailability and reduce acute oral toxicity by providing sustained release and protecting the payload from rapid systemic clearance. Consequently, such systems allow for effective therapeutic outcomes at significantly lower doses compared to free formulations. In addition, the nanocarrier design is shown in Fig. (5).

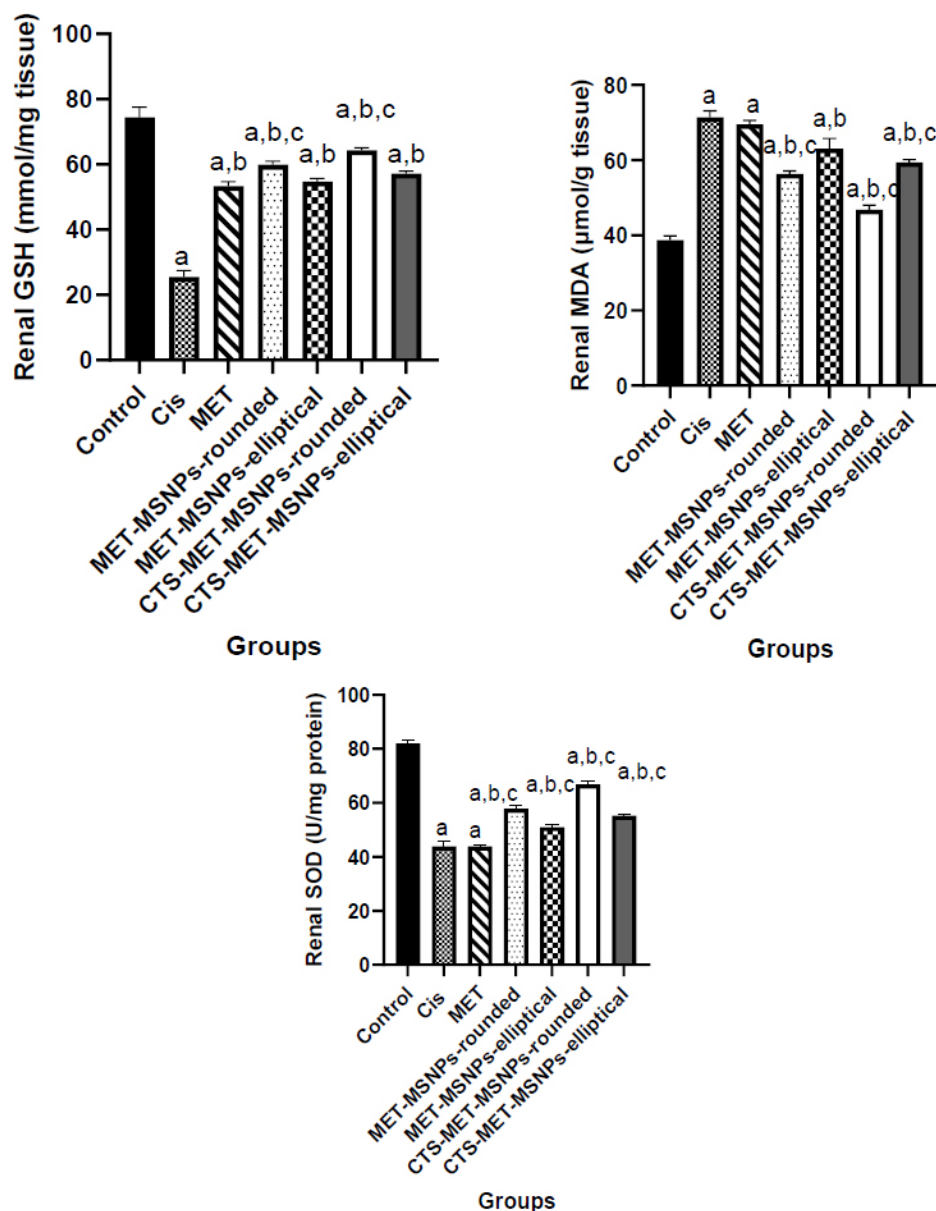


Fig. (5). Levels of Malondialdehyde (MDA), Superoxide Dismutase (SOD), and Reduced Glutathione (GSH) in kidney tissue homogenates. Data are presented as mean $\pm$ SEM (n=5). Statistical significance was determined by Tukey's post-hoc test: ^a $P < 0.05$ vs. control group, ^b $P < 0.05$ vs. cisplatin (Cis) group, ^c $P < 0.05$ vs. free MET-treated group. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.4.3. MET and CTS-MET-MSN Mitigate the Elevation of the Inflammatory Cytokines and Apoptotic Factors; TGF- β 1, TNF- α , IL-1 β and Caspase-3

In comparison with the control group (TNF- α : 24.8 $\pm$ 0.6 pg/mg; IL-1 β : 45.2 $\pm$ 0.4 pg/mg; TGF- β 1: 1.35 $\pm$ 0.14 ng/mg), the levels of TGF- β 1, TNF- α , and IL-1 β were significantly elevated in the Cis group (2.7 $\pm$ 0.1 ng/mg, 121.3 $\pm$ 3.1 pg/mg, and 122.3 $\pm$ 2.8 pg/mg, respectively). However, the alterations in the levels of in-

flammatory mediators in the MET-treated group showed substantially reduced levels when compared to the Cis group. Administration of rounded CTS-MET-MSNPs caused a significant suppression in inflammatory cytokine levels ($P < 0.05$) compared to the Cis and MET-treated groups (Fig. 6), with a better effect for rounded nanoparticles relative to elliptical ones; specifically, rounded CTS-MET-MSNPs reduced TNF- α to 52.8 $\pm$ 1.8 pg/mg, representing a 56% decrease compared to the Cis group.

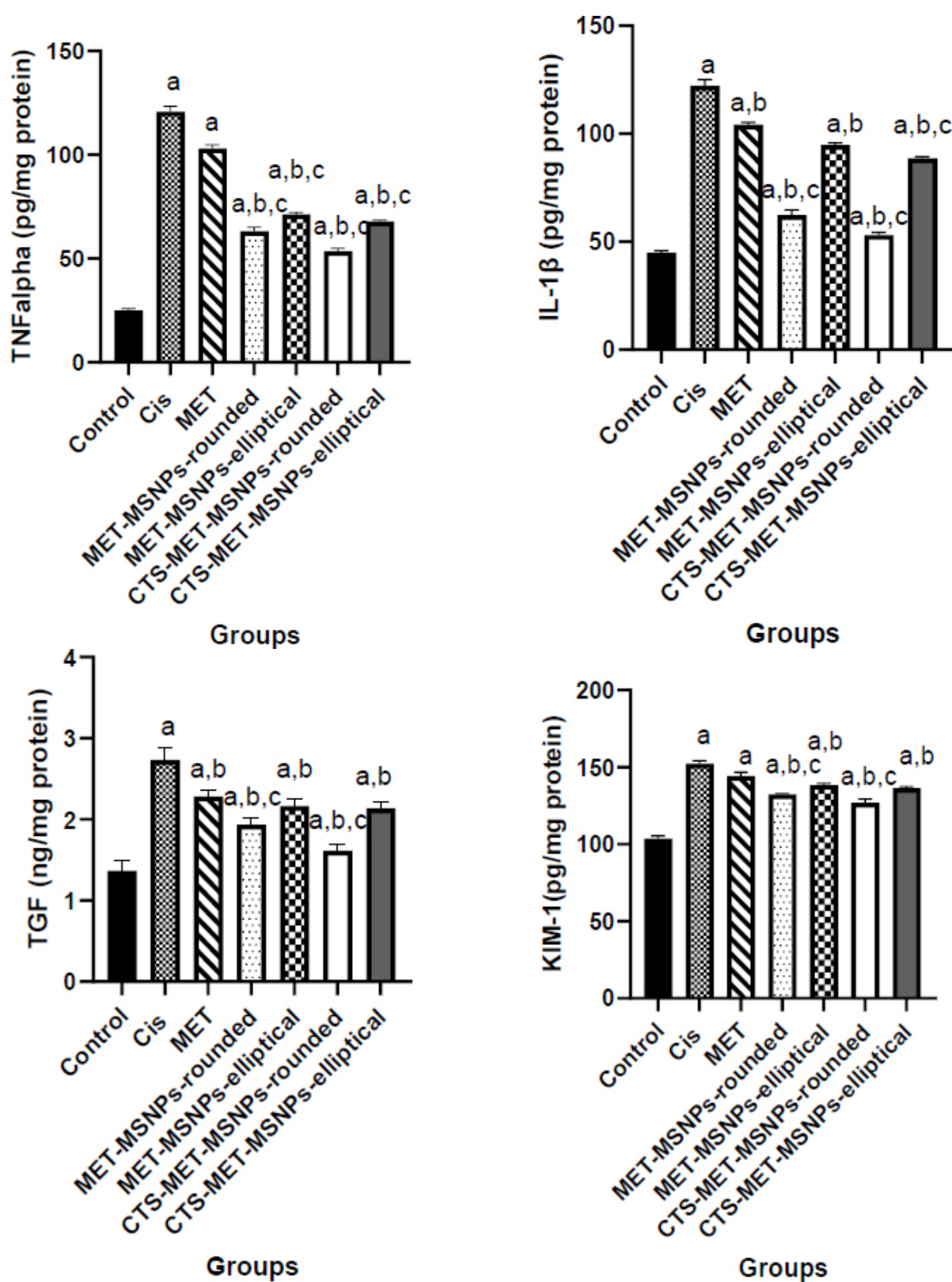


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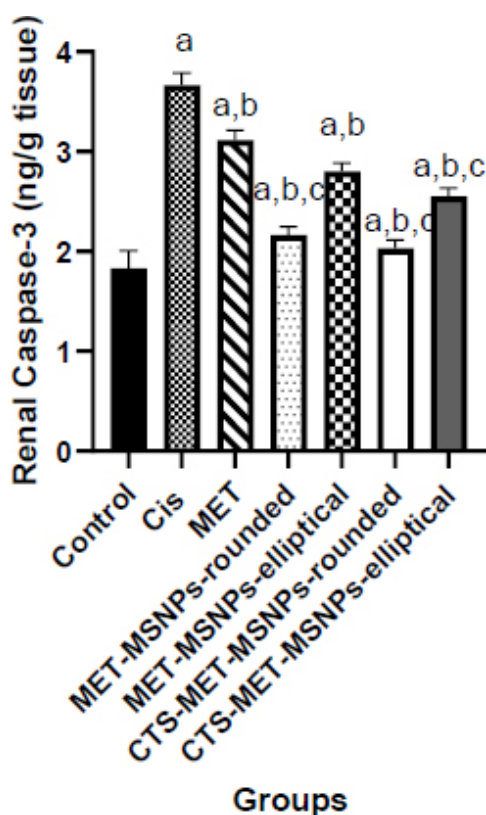


Fig. (6). TGF- β 1, TNF- α , IL-1 β , Caspase-3, and KIM-1 levels were quantified *via* ELISA. Data are mean $\pm$ SEM (n=5). Statistical significance was determined by Tukey's test: ^a $P < 0.05$ vs. control group, ^b $P < 0.05$ vs. cisplatin (Cis) group, ^c $P < 0.05$ vs. free MET-treated group. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

In order to investigate the apoptotic effect of Cis, we assayed the content of caspase-3 in renal tissue using the ELISA technique, where Cis caused a higher elevation of the caspase-3 level (3.6 ± 0.14 ng/g) compared to the control group (1.8 ± 0.19 ng/g). Remarkably, a significant anti-apoptotic activity was observed in the Cis + CTS-MET-MSNPs rounded nanoparticles group (2.0 ± 0.09 ng/g) relative to the Cis + MET-MSNPs elliptical nanoparticles group (2.8 ± 0.08 ng/g; $P < 0.05$). Administration of either MET or MET-MSNPs alone also reduced the level of caspase-3 significantly relative to the Cis group. Additionally, CTS-MET-MSNPs extensively reduced KIM-1 levels when administered in combination with Cis ($P < 0.05$), reaching 126.4 ± 2.8 pg/mg for the rounded CTS form—and caused better activity relative to the Cis + MET group (144.5 ± 2.6 pg/mg; $P < 0.05$). Unexpectedly, rats treated with either rounded MET-MSNPs or the elliptical form at the same dose revealed a significant reduction in KIM-1 levels relative to the Cis group ($P < 0.05$). These results highlighted the enhanced protective activity of MET in both the free form and rounded loaded nanoforms.

3.4.4. The effect of MET and CTS-MET-MSN on the AMPK/PI3K/Akt and mTOR signaling pathways in renal tissue

Fig. (7) showed that the gene expression of AMPK, PI3K and Akt were significantly activated after Cis intoxication ($P < 0.05$), reaching relative expression levels of 4.12 ± 0.12 , 4.84 ± 0.11 , and 4.42 ± 0.12 , respectively, compared to the control group (0.72 ± 0.17 , 0.85 ± 0.10 , and 1.18 ± 0.10 , respectively). Remarkably, the gene expression of the mammalian target of rapamycin (mTOR) was reduced in the tissue of the Cis group (1.08 ± 0.14) relative to control rats (3.81 ± 0.10). Additionally, the administration of MET and CTS-MET-MSN alleviated the toxicity of Cis and showed a significant reduction ($P < 0.05$) in the gene expression of AMPK/PI3K/Akt—with free MET reaching 3.24 ± 0.16 , 4.11 ± 0.09 , and 4.23 ± 0.15 , respectively—alleviating this signaling pathway as well as enhancing the expression of mTOR. It is worth mentioning that rounded CTS-MET-MSN showed a substantial reduction in the genetic expression of this signaling pathway relative to the MET group. It is obvious in our study that rounded CTS-MET-MSN showed a substantial reduc-

tion ($P < 0.05$) in the expression of the AMPK/PI3K/Akt pathway (1.48 ± 0.07 , 2.38 ± 0.14 , and 1.89 ± 0.06 , respectively) relative to the elliptical nanoparticles (2.56 ± 0.10 , 3.45 ± 0.07 , and 3.27 ± 0.19 , re-

spectively), indicating its antiapoptotic and autophagy regulation capabilities. Furthermore, rounded CTS-MET-MSNPs showed the highest efficacy in restoring mTOR levels to 3.35 ± 0.09 , representing a significant recovery in effect magnitude.

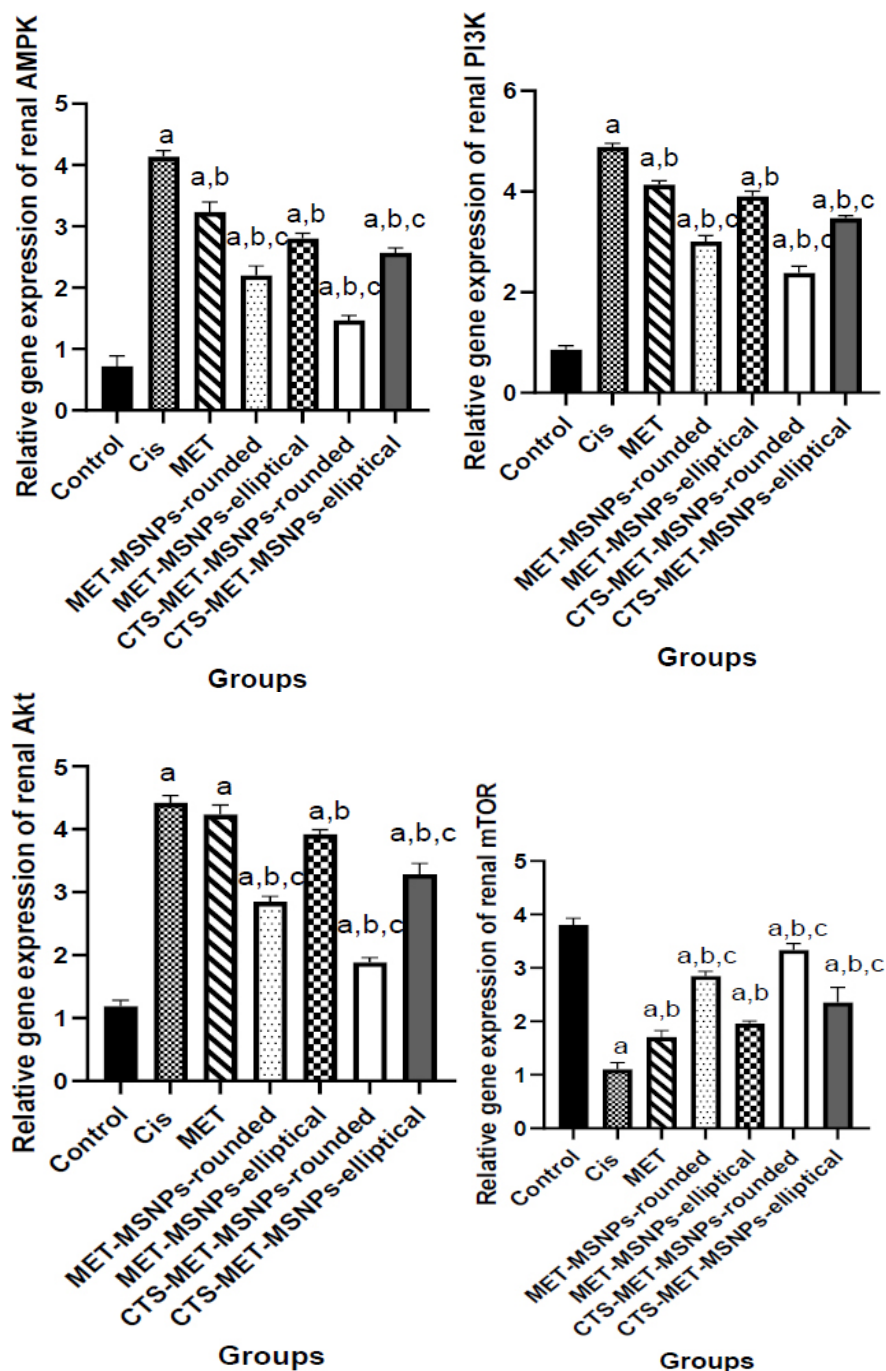


Fig. (7). Relative mRNA levels were determined via qPCR and normalized to β -actin using the $2^{-\Delta\Delta Ct}$ method. Data are mean $\pm$ SEM (n=5). Statistical significance: ^a $P < 0.05$ vs. control group, ^b $P < 0.05$ vs. cisplatin (Cis), ^c $P < 0.05$ vs. free MET-treated group. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.5. MET and CTS-MET-MSNP Protect the Histological Structure of the Kidney Tissue

Histopathological changes of the kidneys of rats were considered to investigate the ability of MET, either free or in loaded nanoform to protect the kidneys from the damaging effects of Cis. Examination under the microscope of renal tissues in the control group showed normal histological structure of both renal cortex and medulla (Fig. 8A). The rat intoxicated with Cis showed mononuclear inflammatory cell infiltration and fibroplasia in the renal cortex. Renal tubular degeneration and necrosis were frequently observed. Some of the examined sections showed marked hemorrhage with renal tubular necrosis (Fig. 8B). Moreover, the kidneys of rats treated with MET exhibited moderate improvement; some of the examined sections showed small multifocal aggregations of mononuclear inflammatory cells in both renal cortex and medulla. In other groups of rat kidneys treated with rounded and elliptical MET-MSNP, the kidney tissue sections showed moderate protection from cisplatin-induced renal damage, as the renal tubular degeneration was alleviated with the presence of small focal aggregates of mononuclear inflammatory cells in some instances (Fig. 8D and E). In comparison, CTS-MET-MSNP treatment revealed a marked protective action was noticed as most of the examined sections were apparently normal, with a more significant protective effect for rounded nanoparticles relative to elliptical ones (Fig. 8F and G).

3.6. MET and CTS-MET-MSNP Attenuate p53 Gene Expression in Kidney Tissue

p53 is a tumor suppressor protein that exerts an important role in the regulation of apoptosis. Cis induced a greater elevation in the expression of p53 when compared to the control group (Fig. 9). To confirm the effect of MET on the changes induced in the expression of p53, we further investigated the expression of p53, which decreased with MET administration. Additionally, the level of p53 gene expression in the rounded CTS-MET-MSNP group is significantly reduced relative to the MET and elliptical CTS-MET-MSNP group ($P < 0.05$). Consequently, the antiapoptotic effect of kidney tissue was investigated by immunohistochemical staining. Consequently, the results indicated that MET in both free form and rounded-loaded nanoforms attenuated apoptosis under Cis exposure.

4. DISCUSSION

Metformin is one of the most common treatments for type 2 diabetic patients; it may reduce carcinogenesis /nephrotoxicity *via* activation of the AMPK signal-

ing pathway. Additionally, previous studies revealed that metformin can stop cell apoptosis through autophagy induction in cell culture of tubular cells [38]. Thus, it is possible that metformin can protect against cisplatin-induced nephrotoxicity by activating autophagy in tubular epithelial cells.

Cisplatin is known as the most common anticancer drug in treating many cancers; however, its side effects on several organs have restricted its application [39]. Kidney dysfunction is the most common side effect of treatment with Cis [40]. In this study, we evaluated the damaging activity of Cis on the kidney and the possible effect of MET in both free form and different loaded nano-formulations. Chitosan is a natural polysaccharide; it is known for its safety due to its biocompatible and biodegradable effects, which increase its medical uses in drug delivery forms [41].

The design of the CTS-MET-MSNP carrier in our study followed well-established principles, aiming to control enhanced tissue interaction and renal targeting. CTS-MET-MSNP formulations revealed larger particle sizes, with the rounded configuration reaching approximately 103 nm, likely due to the chitosan coating. Given that nanoparticles slightly above 100 nm can access the kidneys through secretion from peritubular capillaries into the proximal tubules, this size range may support renal delivery potential. The higher PDI values for CTS-MET-MSNP nanoparticles revealed a wider particle size distribution. These results are consistent with previous reports by [42], reflecting the gradual increase in the hydrodynamic diameter after the chitosan coating of MSNPs. This chitosan layer contributes to the overall nanoparticle size *via* the volume of the coating itself. Additionally, chitosan-coated formulations showed an increased PDI compared to their uncoated counterparts [43]. Loading MET onto MSNPs decreased their negative surface charge due to the cationic nature of MET. These findings were confirmed by previous reports by [44], showing that the surface modification of MSNPs with chitosan can cause a shift in the zeta potential from negative to positive values. This positive surface charge not only occurs due to colloidal stability but also improves interaction with the negatively charged renal membranes and the glomerular capsule, thereby improving kidney uptake [45]. The rounded geometry of both MET-MSNP and CTS-MET-MSNP nanoparticles revealed improved entrapment efficiency compared to the elliptical ones that was confirmed by other reports showing higher pore accessibility compared to the elongated ones [46]. The lower entrapment efficiency (~10%) of the elliptical variant may be attributed to geometric

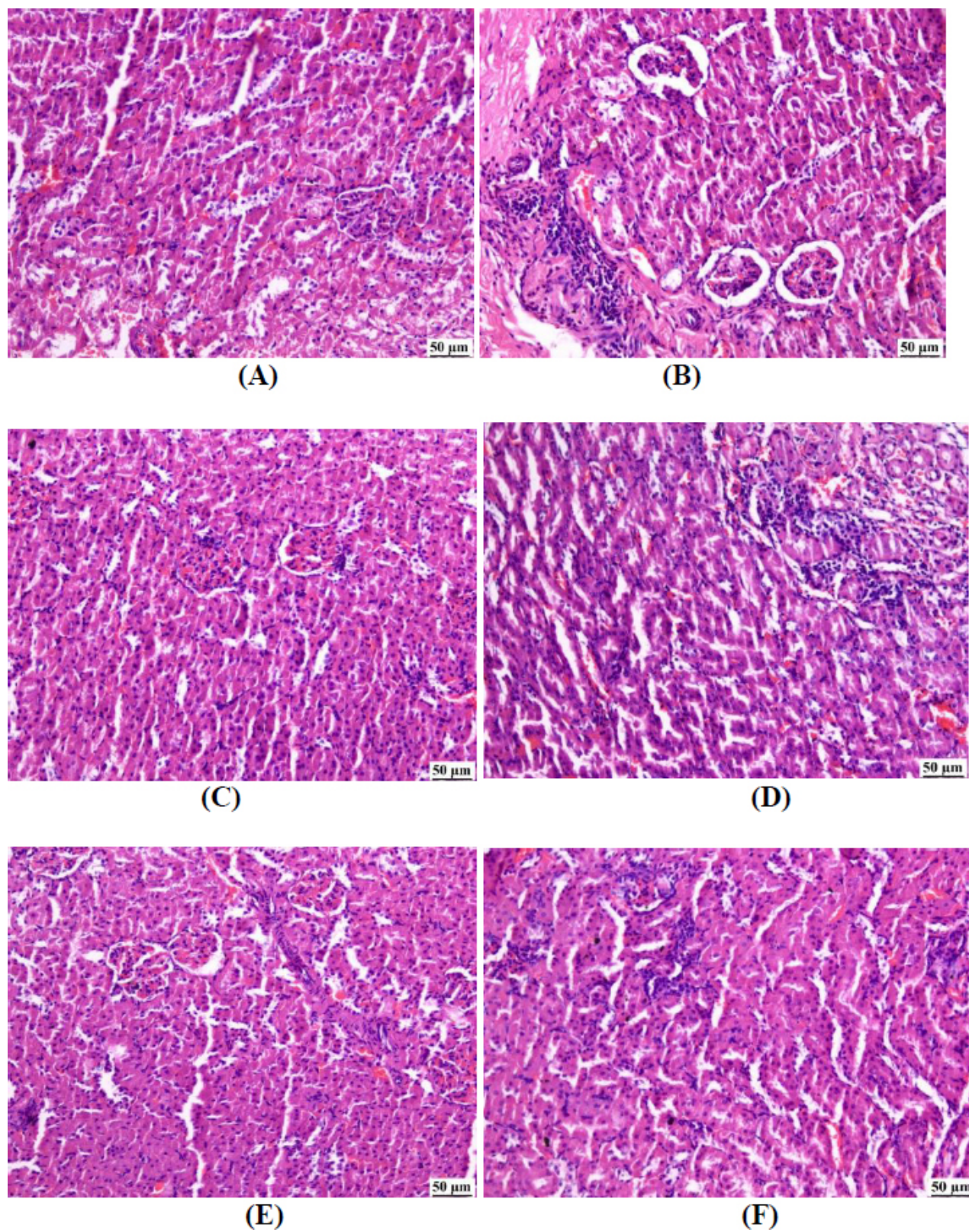


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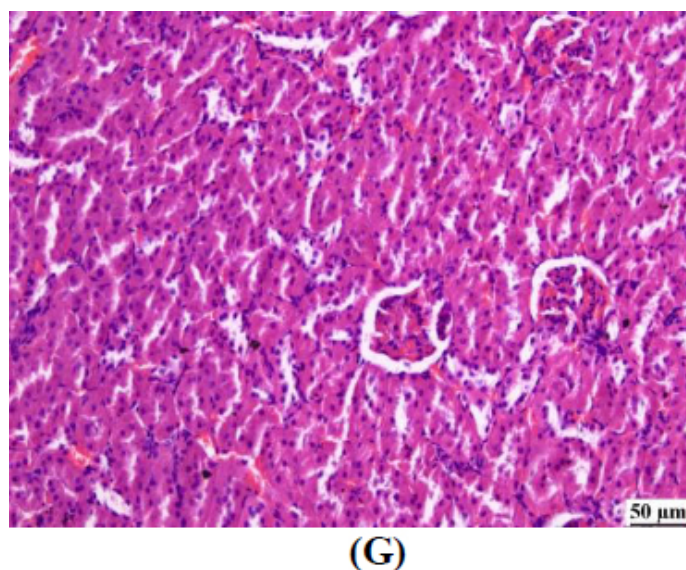


Fig. (8). (A) Control group (normal structure). (B) Cis group (necrosis, hemorrhage, and inflammation). (C) Cis + MET group (moderate improvement). (D, E) MET-MSN rounded/elliptical groups. (F, G) CTS-MET-MSN elliptical/rounded groups (marked protection). Images were captured at 400x magnification. (A); The kidney of a control rat showed normal structure (H and E), (B) The kidney of a cisplatin-treated rat presented mononuclear inflammatory cell infiltration and fibroplasia in the renal cortex. Renal tubular degeneration and necrosis were frequently observed. Some of the examined sections showed marked hemorrhage with renal tubular necrosis (H and E), (C) Moreover, the kidneys of rats treated with MET exhibited moderate improvement, some of the examined sections showed small multifocal aggregations of mononuclear inflammatory cells in both renal cortex and medu (H and E $\times 400$), (D and E) the kidney tissue sections treated with rounded and elliptical MET-MSN showed moderate protection from cisplatin induced renal damage as the renal tubular degeneration was alleviated with existence of small focal aggregates of mononuclear inflammatory cells in some instances. (F) CTS-MET-MSN-elliptical treatment revealed marked protective action was noticed as most of the examined sections were apparently normal (H and E), and (G) The kidney of the CTS-MET-MSN-rounded treated rat showed normal renal tissues with congested intertubular blood vessels with albuminous casts in the renal tubules (H and E). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

constraints and potential 'pore-end blocking' during the loading process, which restricts drug diffusion into the internal mesoporous channels. Beyond loading capacity, particle shape also plays an important role in biodistribution and kidney targeting. Literature reports indicate that rounded and short rod-shaped MSNs accumulate more effectively in renal tissues than elongated or irregular morphologies, largely due to favorable hydrodynamic behavior and enhanced uptake within the renal microenvironment. In this study, a 5-fold reduction in the metformin dose (40\ mg/kg) was employed for the nano-formulations compared to the free metformin group (200\ mg/kg). While direct pharmacokinetic profiles were not established, the significantly enhanced renoprotective effects observed at this lower dose, evidenced by superior reductions in KIM-1 and pro-inflammatory markers, demonstrate a potent dose-sparing effect. This is likely attributed to the mesoporous structure and chitosan coating, which protect the cargo from premature degradation and facilitate targeted in-

teraction with the renal glycocalyx, thereby increasing the effective concentration at the site of injury despite the lower systemic dose. Although the free MET group received 200 mg/kg/day, the NP-treated groups received a significantly lower metformin equivalent (40 mg/kg/day), justified by the enhanced bioavailability, targeted renal delivery, and sustained release characteristics of the MSNP and CTS-MSN nanocarriers. This reduced dose is consistent with previous reports demonstrating superior pharmacokinetic performance of nanoengineered metformin formulations [47]. Blank (drug-free) MSNPs and CTS-MSNPs were not included as a separate group because previous reports indicate good biocompatibility of such carriers. For instance, MacCuaig *et al.* demonstrated minimal *in vivo* toxicity of 25 nm chitosan-coated MSNs after repeated injections in mice [48]. *In vitro* work by Zhang *et al.* found no significant cytotoxicity for Cetuximab-modified silica nanoparticles loaded with ICG in breast cancer cells [49].

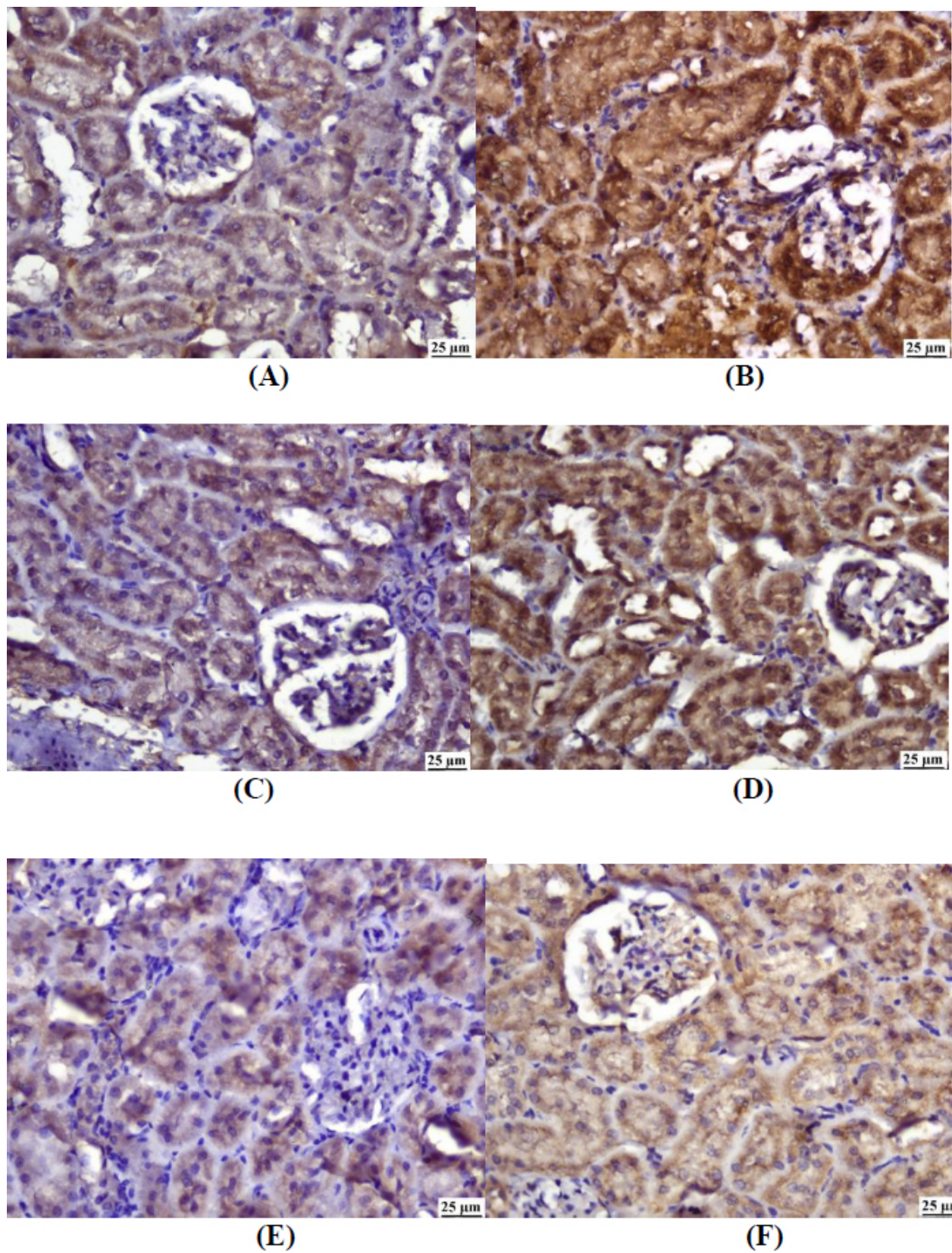
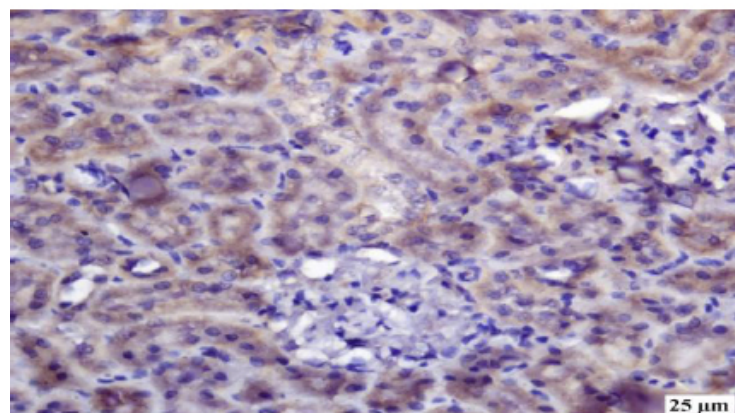
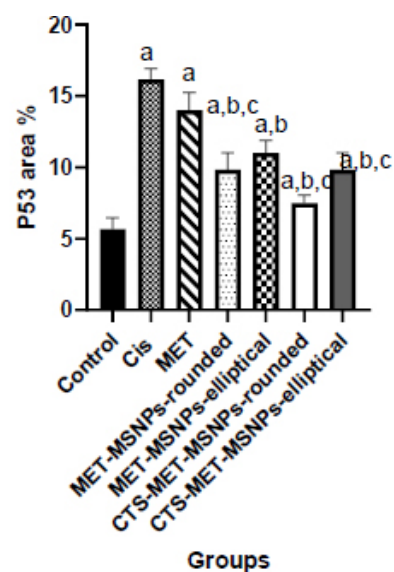


Fig. (9). Contd....



(G)



(H)

Fig. (9). Panels (A–G) show p53-positive immunoreactivity (brown staining) across groups (Scale bar = 100 μ m). Panel (H) represents the quantitative area % of p53 expression. Data are mean $\pm$ SEM (n=5). Statistical significance was determined by Tukey's post-hoc test: ^a $P < 0.05$ vs. control group, ^b $P < 0.05$ vs. cisplatin (Cis), ^c $P < 0.05$ vs. free MET-treated group. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

In light of the available evidence, the design of our carrier system was guided by key parameters known to enhance kidney targeting. Rounded nanoparticles were selected since their geometry has been reported to promote efficient circulation, facilitate passage through the glomerular filtration barrier, and improve interaction with renal tissues. In addition, surface functionalization with chitosan was introduced to confer a positive charge, which is expected to favor electrostatic interactions with the negatively charged renal cell membranes. Accordingly, our formulation strategy—rounded particles with CH coating—was deliberately chosen to promote potential renal selectivity. The morphological assessment of CTS-MET-MSNPs by TEM showed the successful formulation of the rounded nanoparticles with a defined mesoporous structure. After chitosan coating, a thin polymeric layer was observed on the particle surface that blocked the pores, reflecting the effective adsorption of chitosan onto the silica matrix *via* charge interactions and hydrogen bonding. The FTIR spectra showed characteristic bands corresponding to chitosan ($-\text{NH}_2$ and $-\text{OH}$ stretching vibrations) together with the Si-O-Si stretching of the silica formulation.

In terms of release behavior, the release of MET from MET-MSNPs showed biphasic release profiles with an initial burst release followed by sustained re-

lease controlled primarily by diffusion from the porous matrix [50]. Coating the MET-MSNPs with chitosan exhibited an extended-release profile at pH 7.4 relative to pure drug. The hydrophilic nature of chitosan facilitates the production of a hydrated gel-like barrier after exposure to the aqueous medium that reduces drug mobility and release, confirming the role of hydration of the polymer in controlling diffusion rates [51].

In line with these results, the in-vitro release profiles of MET from different formulations were fitted to some kinetic models to investigate the release mechanism. The release of MET from MET-MSNPs showed a biphasic profile with an initial burst followed by a slow sustained release that fitted with the Higuchi model ($R^2 = 0.71$ at pH 7.4 and 0.63 at pH 5.5), which confirms that the release process was diffusion-driven *via* the mesoporous framework. However, a best fit was obtained from the Korsmeyer-Peppas model ($R^2 = 0.96$ at pH 7.4 and 0.94 at pH 5.5), showing that MET release followed an anomalous mechanism. On the contrary, the release behavior of CTS-MET-MSNPs was better indicated by the Higuchi ($R^2 = 0.95$ at pH 7.4 and 0.86 at pH 5.5) model, revealing a slightly superior correlation. The chitosan coating played an essential role in sustaining release, which is consistent with previous studies on chitosan-modified mesoporous carriers [52].

Beyond the physicochemical modifications, the biological relevance of the MET-MSNP system became evident upon coating with chitosan, which further influenced its protective role against cisplatin-induced toxicity. Subsequently, the coating of MET-MSNP with chitosan was effectively able to mitigate the damaging properties of Cis. The prevalence of kidney toxicity after induction with Cis mainly occurred due to inhibition of the defense mechanism and production of ROS. These ROS control several signaling pathways, including cellular necrosis. Additionally, Accumulation of ROS in the rat's kidney tissue treated with Cis inhibited the antioxidant mitochondrial competence, leading to elevated apoptosis of renal tissues [53]. This hypothesis is indicated in our present study by elevation of MDA and reduction of SOD and GSH levels in the kidneys of the Cis group. Administration of MET in its free or nano-formulation form to rats induced by Cis didn't restore significantly the MDA, SOD, and GSH levels. MET is considered a potential scavenger for free radicals [54]. Notably, CTS-MET-MSNP demonstrated a significantly greater antioxidant effect compared with both free MET and MET-MSNP. This enhanced activity may be attributed to multiple synergistic mechanisms. First, surface functionalization with chitosan, a cationic polymer, improves nanoparticle stability, cellular uptake, and tissue adhesion, facilitating enhanced renal tissue interaction. Second, chitosan itself possesses well-documented intrinsic antioxidant and anti-inflammatory properties [55], which may complement the effects of MET. Third, the sustained-release profile of MET from MSNPs ensures prolonged drug availability at the target site, reducing rapid clearance and maintaining effective antioxidant activity. Nephrotoxicity induced by Cis has been confirmed in our study, as verified by an increase in serum creatinine and urea levels. These changes in biochemical markers are related to histopathological investigations. Several histological alterations were detected, including renal tubular degeneration and necrosis. Additionally, renal uptake of cisplatin is regulated *via* several transporters, resulting in a potential reduction in renal function and cell death [56]. High expression of cytokines in our study including IL-1 β , TGF- β 1, TNF, caspase-3 and KIM-1 which is a trans-membrane protein, these markers are considered as early markers for diagnosis of tubular and glomerular injury caused by damaged kidney tubules. Especially, KIM-1 is elevated earlier than other standard markers, including creatinine and urea. This suggests a synergistic effect arising from the combined contributions of metformin's intrinsic bioactivity, chitosan's reported anti-inflammatory/antiapoptotic potential, and the engineered nanopar-

ticle design (geometry + surface functionalization), as also reflected in histopathological improvements. The ability of CTS-MET-MSNP to regulate kidney function is observed by a significant suppression in urea and creatinine levels, together with a potential reduction of IL-1 β , TGF- β 1, and KIM-1 expression induced by Cis. Similarly, MET in free or loaded nanoform downregulated the expression of caspase-3 and TNF- α , with more significant effects observed in CTS-MET-MSNP-treated groups compared to MET and MET-MSNP-treated groups.

To further investigate the downstream consequences of metabolic modulation, we evaluated the expression of P53. While traditionally viewed as a tumor suppressor, P53 serves as a critical node in the AMPK/mTOR signaling network. Furthermore, P53 reinforces AMPK activation through the induction of Sestrins, which inhibit mTORC1 activity *via* the GATOR complex. The upregulation of P53 in our study likely reflects a coordinated cellular response to the modulation of the AMPK/mTOR axis, contributing to the overall therapeutic efficacy of the MET-MSNP formulation in regulating apoptosis. Apoptotic activation is linked to activation of p53 in mitochondria, resulting in activation of caspase-3 and initiation of apoptosis through oxidative stress signaling pathways [57]. Our results showed that cisplatin administration significantly down-regulated mTOR expression, confirming a state of severe cellular exhaustion, compromised protein synthesis, and tubular cell death. Interestingly, treatment with CTS-MET-MSNP significantly restored mTOR levels toward control values by successfully stimulating the AMPK pathway; the nano-formulation orchestrates an immediate cytoprotective autophagic response to clear cisplatin-induced damaged mitochondria and aggregates, which structurally paves the way for the observed downstream recovery of baseline mTOR-mediated cellular proliferation.

In acute kidney injury, basal mTOR activity is essential for renal tubular epithelial cell survival and the initiation of proliferation necessary for tissue repair and regeneration. Therefore, the effect of CTS-MET-MSNP represents a homeostatic restoration rather than a pathological upregulation, balancing AMPK-mediated protective autophagy during acute kidney injury with mTOR-mediated structural recovery during progressive treatment. This dual modulation underlines the superior therapeutic indexing achieved by the nano-formulation [58].

To investigate the mechanistic signaling pathways of MET and CTS-MET-MSNP, immunohistochemistry assessment of renal tissue was performed relative

to Cis-induced apoptosis and different related proteins. Our findings confirmed that Cis-induced renal injury was contributed to by apoptosis. We observed that Cis promoted activation of p53 and caspase-3, thus triggering renal apoptosis. Previous reports revealed that Cis promoted apoptosis in kidneys by reduction of Bcl-2 and elevation of p53 expression [59].

There is clear agreement that Cis upregulated the signaling pathway of caspase-3 and p53, which is confirmed with the present study. The expression of these apoptotic markers was significantly downregulated by CTS-MET-MSNP compared to the Cis-treated group, with potential effects for the rounded form compared to the elliptical ones. This shape-dependent variation is consistent with literature indicating enhanced renal accumulation and cellular uptake of more rounded nanoparticles in certain microvascular beds, potentially explaining the superior bioactivity of the rounded CTS-MET-MSNP in modulating apoptotic signaling. Taken together, the effective protective effect against Cis renal toxicity is significantly attributed to the chitosan-coated nanoformulation of MET compared to the free form [60]. Moreover, MET did not improve GSH and MDA levels compared to CTS-MET-MSNP. Thus, another previous study revealed that the structure of MET should be optimized to develop a new compound with better solubility and bioavailability. The underlying signaling pathways of Cis and MET investigated in the text are shown in our results. Several studies have reported that MET treatment with 500-850 mg of MET daily has beneficial effects without any toxic reactions [61]. Accordingly, CTS-MET-MSNP can be given at lower doses than MET free form to enhance health status.

Previous studies have also revealed that Cis induces apoptosis through the AMPK signaling pathway, which is important in the promotion of renal injury induced by Cis [62, 63]. Additionally, the target of the AMPK signaling pathway is the PI3K/Akt downstream that is correlated with the survival of cells. The activity of this PI3K/Akt pathway in renal injury is debatable; many studies revealed that the activation of PI3K/Akt was correlated with kidney injury and the production of ROS, together with apoptosis after Cis administration [64]. On the contrary, others showed that this signaling pathway was suppressed after Cis administration [65]. Our study showed that Cis treatment induced the elevation of AMPK/PI3K/Akt gene expression in kidney tissue, which was confirmed by previous results [66, 67].

As cell growth is controlled by mTOR, which is controlled through the upstream PI3K/Akt regulator

[68], we found that CTS-MET-MSNP treatment revealed a significant increase in the expression of mTOR compared to MET. Therefore, we revealed that kidney apoptosis induced by Cis could be through the stimulation of AMPK activity and the PI3K/Akt signaling pathway, along with the stimulation of p53 and caspase-3. Obviously, the suppression of AMPK and the elevation of mTOR mitigate the progression of kidney damage that is caused by Cis, which is confirmed by several studies [69].

Collectively, these findings highlight how the rational design of the CTS-MET-MSNP carrier leveraging chitosan's functional properties, metformin's pleiotropic actions, and geometry-dependent biodistribution contributed to the observed renoprotective effects. This approach highlights a nanomedicine perspective that aims to potentiate drug delivery and safety following cisplatin-induced nephrotoxicity.

CONCLUSION

Cisplatin (Cis) induces renal tissue injury primarily through oxidative stress, which activates redox signaling pathways, elevates pro-inflammatory mediators, and triggers apoptosis. In our study, metformin (MET) and its chitosan-coated mesoporous silica nanoparticles (MET-MSNP-CH) revealed superior renoprotective properties against Cis-induced nephrotoxicity. The aim of our design of the nanoparticle system mixing the elevated drug-loading capacity of MSNPs with the selectivity-enhancing and stabilizing properties of chitosan and shape MET's sustained release and bioavailability. This design improved MET's anti-inflammatory, antiapoptotic, and antioxidant properties. Collectively, our results suggest that chitosan-coated MSNPs could be a promising candidate for site-specific delivery nanoform to improve MET's therapeutic efficacy and also provide protection compared to the free MET following the administration of Cis.

LIMITATIONS OF THE STUDY

Despite the promising findings of the present study, several limitations should be acknowledged. First, the study was conducted using a single animal species (Wistar rats) and a single sex (male), which limits the generalizability of the results, as sex-specific differences in cisplatin-induced nephrotoxicity and metformin pharmacodynamics have been reported. Blank nanocarrier groups were not included; therefore, potential off-target effects of the carrier alone cannot be fully excluded. Future studies incorporating female animals and additional species would be valuable to improve translational relevance. Second; long-term toxic-

ty, and recovery outcomes need further investigation to better define therapeutic windows and safety margins, particularly for nanoparticle-based formulations. Future studies incorporating PK modeling, female animals, and additional species would be valuable to improve translational relevance. Second, long-term toxicity and recovery outcomes need further investigation to better define therapeutic windows and safety margins, particularly for nanoparticle-based formulations. Finally, addressing risk of bias and experimental conduct. Despite these controls, residual sources of bias inherent to preclinical animal studies, including inter-individual biological variability and laboratory-specific conditions, need further investigation. Addressing these points in future research will promote the impact and applicability of the outcomes of the study.

AUTHORS' CONTRIBUTIONS

OF and SH participated in the study design, practical work, especially the molecular biology, as well as interpretation of data and manuscript preparation. SH and MS participated in practical work, interpretation of data, and manuscript preparation. All authors read and approved the manuscript, and all data were generated in-house; no paper mill was used.

LIST OF ABBREVIATIONS

AMPK	= Activated Protein Kinase
cDNA	= Complementary DNA
Cis	= Cisplatin
CTS- MET	= Chitosan-coated Metformin MSNPs
-MSNPs	
DLS	= Dynamic Light Scattering
EE%	= Encapsulation Efficiency
FTIR	= Fourier Transform Infrared Spectroscopy
GSH	= Glutathione
IL-1 β	= Interleukin-1 β
mTOR	= Mammalian Target of Rapamycin
MET	= Metformin
MSNs	= Mesoporous Silica Nanoparticles
MDA	= Malondialdehyde
NF- κ B	= Nuclear Factor Kappa B
PS	= Particle Size
PDI	= Polydispersity Index

ROS	= Reactive Oxygen Species
RT	= Reverse Transcriptase
GSH	= Reduced Glutathione
qPCR	= Real-time Polymerase Chain Reaction
SOD	= Superoxide Dismutase
TGF β 1	= Tumor Growth Factor
TNF- α	= Tumor Necrosis Factor
TEM	= Transmission Electron Microscope
TBA	= Thiobarbituric Acid
ZP	= Zeta Potential

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Ethics Committee of the Faculty of Pharmacy, MSA University (code: PB37/REC37/2024P).

HUMAN AND ANIMAL RIGHTS

This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All the data and supporting material are available within the article.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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