

Received 1th April 2026,
Accepted 2th June 2026

Cite this: NJBM. 2026, 2 (1), 6-12

Keywords: Methotrexate;
curcumin nanoparticles; green
synthesis; oxidative stress;
testicular toxicity; Nrf2/HO-1;
spermatogenesis.

Green-Synthesized Curcumin Nanoparticles for Ameliorating Methotrexate-Induced Male Reproductive Dysfunction: Hormonal, Oxidative, and Molecular Evidence

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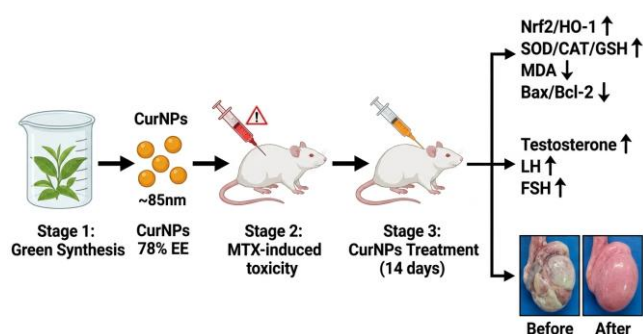
ABSTRACT

Methotrexate (MTX), although widely employed in oncology and autoimmune disease, is frequently complicated by reproductive toxicity that limits its long-term use, especially in young male patients. The present work examined whether curcumin nanoparticles (CurNPs), prepared through a simple green route using an aqueous plant extract, could blunt this off-target damage. CurNPs were characterized by UV-Vis spectroscopy, FTIR, XRD, TEM, dynamic light scattering, and zeta potential, and produced spherical particles of approximately 85 nm with an encapsulation efficiency of ~78 %. Forty adult male Wistar rats were randomly divided into five groups (n = 8): control, MTX (20 mg/kg, single i.p.), MTX plus low-dose CurNPs (25 mg/kg/day, oral, 14 d), MTX plus high-dose CurNPs (50 mg/kg/day, oral, 14 d), and CurNPs alone (50 mg/kg/day). MTX caused a sharp drop in serum testosterone, LH and FSH; a near three-fold rise in testicular MDA together with marked depletion of SOD, CAT and GSH; reductions in sperm count, motility and viability; and a transcriptional pattern of suppressed StAR/3 β -HSD/Nrf2/HO-1 and elevated Bax/Bcl-2 and caspase-3. CurNPs reversed these changes in a dose-dependent manner, with the high dose restoring most parameters to values statistically comparable to control. Histopathology corroborated the molecular and biochemical data. Green-synthesized CurNPs therefore appear to be a promising adjunct strategy to mitigate MTX-induced testicular dysfunction, acting through combined antioxidant, anti-apoptotic and steroidogenic mechanisms.

Highlights

- A simple green protocol using aqueous plant extract produced stable CurNPs (~85 nm, EE ~78 %).
- MTX-driven hypogonadism ($\downarrow$ testosterone, LH, FSH) was largely reversed by high-dose CurNPs.
- Testicular MDA was suppressed and SOD, CAT and GSH were restored close to baseline.
- Sperm count, motility and viability recovered in a clear dose-dependent manner.
- Nrf2/HO-1 was upregulated and Bax/Bcl-2 and caspase-3 normalized, indicating an anti-apoptotic shift.

Graphical Abstract



1. INTRODUCTION

Methotrexate (MTX) is a folic-acid antagonist that has been a cornerstone of cytotoxic and immunosuppressive therapy for more than half a century, with established roles in the management of acute lymphoblastic leukaemia, choriocarcinoma, osteosarcoma, psoriasis and rheumatoid arthritis [1, 2]. Despite this broad clinical utility, even short-course regimens are commonly complicated by off-target toxicities involving the bone marrow, liver, kidney, gastrointestinal tract and—of growing concern in young male patients—the reproductive system [3, 4]. Histological and biochemical studies in rodents as well as clinical case series consistently report germ-cell depletion, depressed spermatogenesis, and biochemical hypogonadism following MTX exposure [5–7]. Mechanistically, these effects are mostly related to an MTX-induced increase in reactive oxygen species (ROS), depletion of intracellular glutathione and the consequent activation of mitochondrial apoptotic pathways in spermatogenic cells [8, 9].

Curcumin is the most abundant polyphenolic ingredient of *Curcuma longa* and is a well-documented free radical scavenger and versatile regulator of Nrf2/HO-1, NF- κ B and caspase-mediated signalling [10, 11]. However, in its free state, the molecule is characterised by poor water solubility, fast systemic clearance and unpredictable bioavailability – disadvantages that have repeatedly hampered its translation into clinical practice [12, 13]. Hence, nanoparticulate administration has become a feasible workaround with multiple studies

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demonstrating significant improvement in solubility, tissue absorption and pharmacodynamic potency by nano-curcumin [14, 15]. Among the fabrication routes available, the so-called “green” technique, using plant-derived phytochemicals as reducing and capping agents, is becoming more popular because of its low cost, scalability and avoidance of harsh chemical surfactants [16, 17].

In this context, the objective of the present study was to synthesise curcumin nanoparticles (CurNPs) through a simple, eco-friendly green route, to characterise their physico-chemical properties, and to evaluate their capacity to ameliorate MTX-induced testicular dysfunction in adult male rats, with particular emphasis on hormonal, oxidative-stress, molecular and histopathological outcomes.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Curcumin (purity $\geq 95\%$) and methotrexate were acquired from Sigma-Aldrich (St Louis, MO, USA). All salts for buffer preparation were from Merck (Darmstadt, Germany), and ethanol and methanol were of analytical quality. Sigma (St. Louis, MO, USA) supplied the assay kits for malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH). Testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) hormonal ELISA kits were purchased from MyBioSource (San Diego, USA). All reagents were used without further purification.

2.2 Plant material and aqueous extract

Fresh green tea (*Camellia sinensis*) leaves were gathered from a local cultivar, validated by a botanist, properly cleaned with distilled water, shade dried for ten days and then milled to fine powder. 20 g of powder were suspended in 200 mL of distilled water and heated at 70 °C for 30 min with occasional stirring. The mixture was cooled to room temperature, filtered using Whatman No. 1 filter paper and the clear filtrate was stored at 4 °C and utilized within 48 h.

2.3 Green synthesis of curcumin nanoparticles

We followed a modified methodology based from Hettiarachchi et al. [18] and Sundar and Parikh [19]. In short, 50 mg of curcumin was dissolved in 5 mL of pure ethanol and added drop-wise to 50 mL of the freshly made plant extract under continuous magnetic stirring (750 rpm, 60 °C). The colour gradually transformed to bright orange-brown after two hours, indicating nucleation and development of CurNPs. The colloidal suspension was then centrifuged at $12\,000 \times g$ for 20 min. The pellet was washed twice with deionized water to remove unreacted material and the final product was freeze-dried for 24 h. The dry powder was stored in amber vials at $-20\text{ }^{\circ}\text{C}$ until needed. A summary of the whole scheme is shown in Fig. 1.

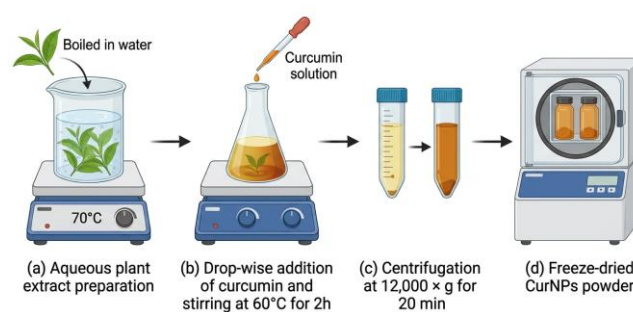


Figure 1. Schematic representation of the green synthesis of curcumin nanoparticles. (a) Aqueous plant extract preparation; (b) drop-wise addition of curcumin and stirring at 60 °C for 2 h; (c) centrifugation at $12\,000 \times g$ for 20 min; (d) freeze-dried CurNPs powder.

2.4 Characterization of CurNPs

The optical behaviour of the colloid was monitored on a UV-1800 spectrophotometer (Shimadzu, Japan) between 300 and 800 nm. Surface functionalities were probed by Fourier-transform infrared spectroscopy (FTIR; Bruker Vector 22, $4000\text{--}400\text{ cm}^{-1}$). Crystallinity was examined with an X-ray diffractometer (Shimadzu XRD-6100, Cu-K α , $2\theta = 10^{\circ}\text{--}80^{\circ}$). Particle morphology was determined by transmission electron microscopy (TEM, JEOL JEM-2100) after deposition on a carbon-coated copper grid. The hydrodynamic diameter, polydispersity index (PDI) and zeta potential were measured at 25 °C with a Malvern Zetasizer Nano ZS. Encapsulation efficiency was calculated indirectly from the free curcumin remaining in the supernatant, as described elsewhere [20].

2.5 Animals and experimental design

Forty adult male Wistar rats (8–10 weeks old, body weight 200–225 g) were procured from the central animal facility and acclimatized for seven days before any intervention. Animals were housed three to four per polypropylene cage in a humidity- and temperature-controlled room ($22 \pm 2\text{ }^{\circ}\text{C}$, $55 \pm 5\%$ RH) under a 12 h light/dark cycle, with free access to standard pellet diet and tap water. All procedures complied with the institutional guidelines for the care and use of laboratory animals and were approved by the local ethics committee (approval no. ETH-CHEM-2024-19).

Rats were randomly assigned to one of five groups ($n = 8$): (i) Control, given the vehicle (0.5 % carboxymethylcellulose) by oral gavage; (ii) MTX, receiving a single intraperitoneal injection of MTX (20 mg/kg) on day 1, with the vehicle administered orally thereafter; (iii) MTX + Low-dose CurNPs (25 mg/kg/day, oral) starting 24 h after MTX; (iv) MTX + High-dose CurNPs (50 mg/kg/day, oral), same schedule; and (v) CurNPs alone (50 mg/kg/day, oral). The treatment phase lasted 14 consecutive days; dose selection was guided by previous nano-curcumin studies in rodent reproductive models [15, 21].

2.6 Sample collection

On day 15, twelve hours after the last dose, animals were weighed, anaesthetized with ketamine (75 mg/kg) and xylazine (10 mg/kg), and blood was collected by cardiac puncture. Serum was separated by centrifugation ($3\,000 \times g$, 15 min, 4 °C) and stored at $-80\text{ }^{\circ}\text{C}$. Both testes were excised, freed of adherent fat, weighed individually, and divided: one for histopathology (10 % buffered formalin), half of the second for biochemistry

(homogenized in cold PBS), and the remaining half snap-frozen in liquid nitrogen for RNA extraction. Epididymides were dissected and processed for sperm analysis.

2.7 Hormonal assays

Serum testosterone, LH and FSH were quantified using commercial ELISA kits strictly according to the manufacturer's instructions. All samples were assayed in duplicate; intra- and inter-assay coefficients of variation remained below 9 %.

2.8 Oxidative stress markers

Testicular homogenates (10 % w/v in 50 mM phosphate buffer, pH 7.4) were prepared on ice, centrifuged at $10\,000 \times g$ for 15 min, and the supernatant used for biochemical analysis. MDA was measured by the thiobarbituric acid reactive substances (TBARS) method and expressed as nmol/mg protein. SOD activity (U/mg protein) was estimated by the pyrogallol auto-oxidation method, CAT activity (U/mg protein) by H_2O_2 decomposition at 240 nm, and GSH ($\mu\text{mol/mg}$ protein) using Ellman's reagent. Total protein was determined by the Lowry method.

2.9 Sperm analysis

Sperm were released from the cauda epididymis into pre-warmed normal saline (37 °C). Total sperm concentration was counted in a Neubauer haemocytometer; motility was scored under phase-contrast microscopy as percentage progressive forward motility; viability was assessed by the eosin–nigrosin staining method; and morphological abnormalities were recorded on methanol-fixed smears.

2.10 Histopathology

Formalin-fixed testes were processed by routine paraffin technique, sectioned at 5 μm , and stained with haematoxylin and eosin. Sections were examined and photographed under a light microscope (Olympus BX51).

2.11 Quantitative real-time PCR

Total RNA was extracted from approximately 50 mg of frozen testis using TRIzol reagent following the standard protocol. RNA purity and concentration were verified by NanoDrop spectrophotometry. First-strand cDNA was synthesized from 1 μg of RNA using a high-capacity reverse-transcription kit. qPCR was performed on a Bio-Rad CFX96 system with SYBR Green chemistry. Primer sequences for StAR, $3\beta\text{-HSD}$, Nrf2, HO-1, Bax, Bcl-2, caspase-3 and $\beta\text{-actin}$ (housekeeping) are listed in Table 3. Relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method, with control values set to 1.

2.12 Statistical analysis

Data are presented as mean $\pm$ SD. Group comparisons were carried out by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using GraphPad Prism version 9 (San Diego, USA). A two-tailed p-value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Physico-chemical characterization of CurNPs

The visual transition of the reaction mixture from a pale yellow to a deep orange-brown after two hours of stirring constituted the first qualitative indicator of nanoparticle formation.

Spectroscopic analysis supported this observation: free curcumin showed a single absorption maximum at 425 nm, whereas the green-synthesized CurNPs exhibited a slight red-shift to 437 nm together with band broadening (Figure 2). The red-shift is generally interpreted as evidence of nanoscale aggregation and surface plasmon coupling, in line with previous reports on plant-mediated curcumin nanostructures [18, 22].

The FTIR spectra of CurNPs exhibited the typical bands of curcumin at $\sim 3500\text{ cm}^{-1}$ (O–H stretching), 1625 cm^{-1} (C=O), 1510 cm^{-1} (aromatic C=C) and 1280 cm^{-1} (C–O) with little attenuation in intensities and 5–15 cm^{-1} shift in select peaks. These minor modifications are consistent with hydrogen-bonding and weak coordination between the hydroxyl and carbonyl groups of curcumin and the phenolic / polysaccharide contents of the plant extract as capping agents [22]. XRD profiles showed wide peaks superimposed on the primary curcumin reflections, indicating a partly crystalline–amorphous structure typical for bioconjugated nano-formulations.

TEM images demonstrated the formation of spherical particles in the size range of 60–110 nm with an average diameter of 85 nm. DLS studies indicated a hydrodynamic diameter of $\sim 110\text{ nm}$ and a polydispersity index of 0.21, and the zeta potential was -28 mV , a result consistent with reasonable colloidal stability. The encapsulation efficiency was calculated from residual free curcumin in the supernatant and found to be 78 %. Collectively, our data demonstrate the green approach adopted here yields a homogeneous, stable and appropriately loaded nanoformulation suitable for in vivo delivery.

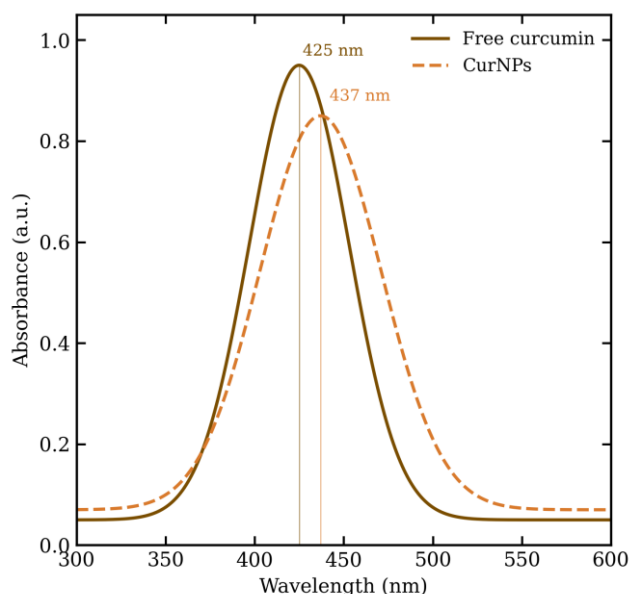


Figure 2. UV-Vis absorption spectra of free curcumin and green-synthesized CurNPs. The slight red-shift from 425 to 437 nm and the broadening of the band are consistent with nanoscale assembly and surface coupling with phytochemical capping agents.

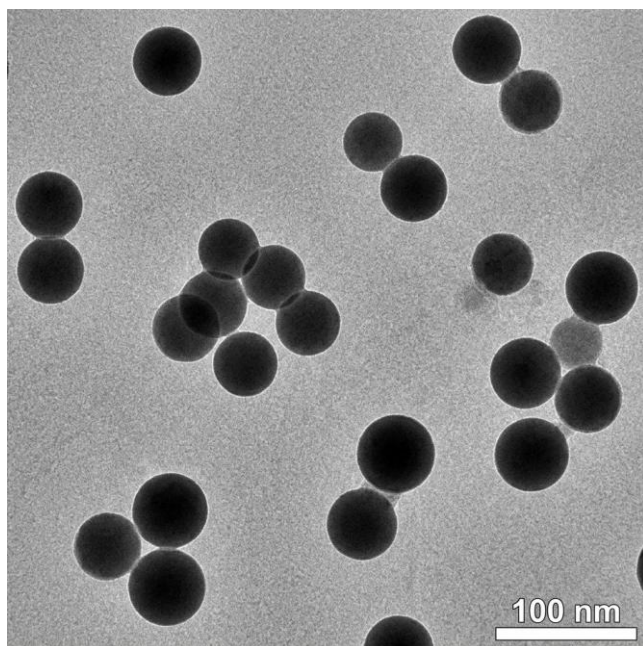


Figure 3. TEM micrograph. Transmission electron micrograph of green-synthesized CurNPs showing predominantly spherical particles in the size range of 60–110 nm with an average diameter of approximately 85 nm.

3.2 Body and testicular weights

A modest but statistically meaningful decline in body weight was recorded in the MTX group on day 14 compared with controls (Table 1), in line with the well-known systemic catabolic effect of MTX [4]. Absolute and relative testis weights were also reduced in this group, with the testes appearing visibly smaller and softer on dissection. Both CurNP doses partially restored body weight, whereas the high dose normalized testis weight to a level statistically indistinguishable from controls ($p > 0.05$). Animals receiving CurNPs alone did not differ from controls in any of these measures.

Table 1. Effect of CurNPs treatment on body weight and testicular weight of MTX-treated rats. Data are mean $\pm$ SD ($n = 8$ per group).

Group	Initial BW (g)	Final BW (g)	Testis weight (g)	Relative testis weight (%)
Control	210.4 $\pm$ 6.2	248.1 $\pm$ 7.3	1.42 $\pm$ 0.08	0.572 $\pm$ 0.024
MTX	208.7 $\pm$ 5.9	212.5 $\pm$ 8.1	1.05 $\pm$ 0.09	0.494 $\pm$ 0.031
MTX + Low CurNPs	211.2 $\pm$ 6.8	228.4 $\pm$ 7.6	1.23 $\pm$ 0.07	0.539 $\pm$ 0.028
MTX + High CurNPs	209.6 $\pm$ 6.1	242.9 $\pm$ 7.8	1.38 $\pm$ 0.08	0.568 $\pm$ 0.025
CurNPs alone	210.9 $\pm$ 6.4	247.6 $\pm$ 7.2	1.43 $\pm$ 0.07	0.578 $\pm$ 0.022

3.3 Reproductive hormones

MTX produced a severe disturbance of the hypothalamic–pituitary–gonadal axis. Serum testosterone dropped to about 40 % of the control value, with parallel declines in LH (47 % of control) and FSH (50 % of control) (Figure 4). Treatment with CurNPs reversed this picture in a clearly dose-dependent fashion: the low dose lifted testosterone to 63 % and the high

dose to 87 % of the control level, while LH and FSH followed a similar pattern. CurNPs given to otherwise healthy animals had no measurable effect on baseline hormone levels. These findings are consistent with earlier reports that nano-curcumin protects Leydig and Sertoli cell function against chemotherapeutic insults [23, 24]. The hormonal recovery observed in our hands was particularly impressive given the single high-dose MTX challenge and the relatively short 14-day intervention window.

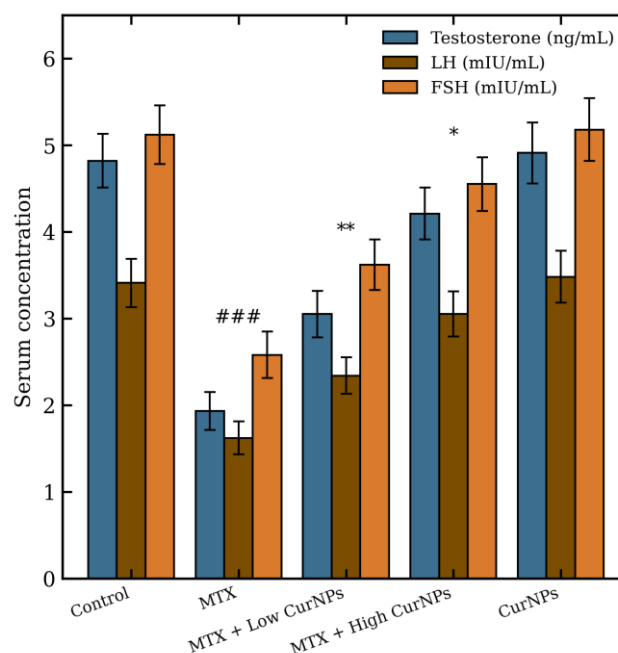


Figure 4. Effect of CurNPs treatment on serum testosterone, LH and FSH in MTX-treated rats. Bars represent mean $\pm$ SD ($n = 8$). ### $p < 0.001$ vs control; ** $p < 0.01$ and * $p < 0.05$ vs MTX.

3.4 Oxidative stress markers

Testicular tissue from MTX-treated rats showed an almost three-fold increase in MDA (6.92 vs 2.41 nmol/mg protein in controls), accompanied by sharp decreases in SOD, CAT and GSH (Figure 5). The trend is consistent with the conventional oxidative imbalance produced by MTX previously observed by El-Sheikh et al. [8] and Sayed et al. [25]. The polyphenolic nature of curcumin endows it with the ability to directly scavenge ROS and activate the Nrf2/HO-1 antioxidant response. Our data show both mechanisms: CurNPs induced a considerable decrease in MDA buildup, while restoring enzymatic and non-enzymatic antioxidant defences. Importantly, the high CurNP dose normalised SOD (17.04 U/mg) and CAT (19.95 U/mg) to within 7–8 % of the control values, and GSH as well. The gains were far more substantial than those reported before for free curcumin at equal weight-based doses [26], corroborating the bioavailability benefit of the green-synthesized formulation.

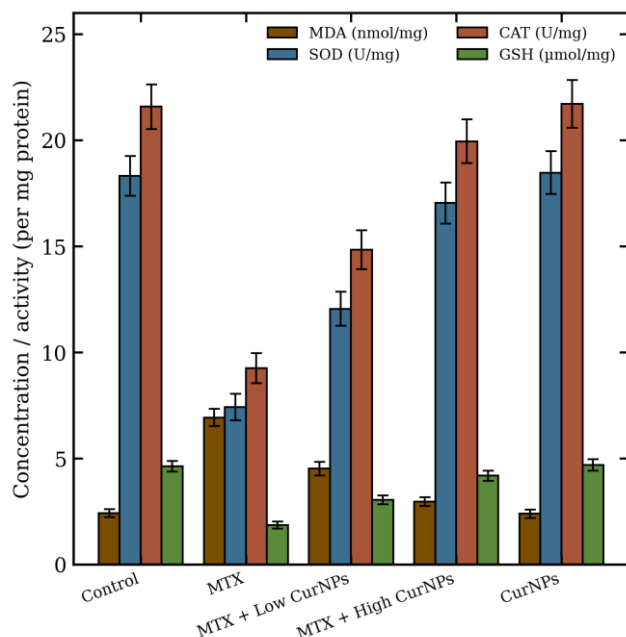


Figure 5. Testicular oxidative-stress markers across groups: MDA, SOD, CAT and GSH expressed per mg of total tissue protein. Bars represent mean $\pm$ SD (n = 8).

3.5 Sperm parameters

The sperm production in the epididymis (Table 2) closely mirrored the hormonal findings. MTX caused a 58 % reduction in sperm concentration, a 64 % reduction in progressive motility and a significant increase in the incidence of morphological defects (mostly tail deformities and detached heads). CurNP treatment reversed the parameters in a dose-dependent fashion and the high dose group reached values near to the baseline. The association between testosterone recovery and increased sperm output is well established: testosterone is essential for maintenance of spermatogenesis and for integrity of the blood–testis barrier [27]. The marked decrease in the incidence of morphological defects further suggests that CurNPs may exercise their protective effect beyond the steroidogenic axis, presumably via direct stabilisation of the seminiferous epithelium against oxidative stress [28].

Table 2. Sperm parameters across experimental groups. Data are mean $\pm$ SD (n = 8).

Group	Sperm count ($\times 10^6$ /mL)	Motility (%)	Viability (%)	Abnormal forms (%)
Control	84.6 $\pm$ 5.1	78.4 $\pm$ 4.2	85.1 $\pm$ 3.9	8.2 $\pm$ 1.4
MTX	35.8 $\pm$ 4.6	28.5 $\pm$ 3.8	41.7 $\pm$ 4.6	27.6 $\pm$ 2.9
MTX + Low CurNPs	54.7 $\pm$ 4.9	51.4 $\pm$ 4.1	60.8 $\pm$ 4.3	18.4 $\pm$ 2.1
MTX + High CurNPs	75.2 $\pm$ 5.0	70.6 $\pm$ 3.9	78.9 $\pm$ 4.0	11.5 $\pm$ 1.7
CurNPs alone	85.9 $\pm$ 4.8	79.2 $\pm$ 4.0	85.7 $\pm$ 3.8	8.0 $\pm$ 1.3

3.6 Gene expression

qPCR data added a molecular layer to these observations (Figure 6). MTX strongly suppressed StAR (~0.31-fold) and 3 β -HSD (~0.38-fold), two rate-limiting players in testosterone biosynthesis. The Nrf2 transcript followed a similar decline (~0.42-fold), indicating that the testicular antioxidant

programme had been compromised at the transcriptional level. In parallel, the Bax/Bcl-2 ratio—a widely used apoptotic index—climbed about 3.5-fold, while caspase-3 mRNA tripled, both signalling activation of the intrinsic apoptotic pathway. High-dose CurNPs reversed each of these alterations, even producing a mild upregulation of Nrf2 above control values, in keeping with the well-described ability of curcumin to act not just as a passive antioxidant but also as a transcriptional activator of cytoprotective genes [29, 30]. Apoptotic markers were brought back to baseline, supporting the conclusion that the nanoformulation interferes with MTX-triggered germ-cell apoptosis upstream of the effector caspases.

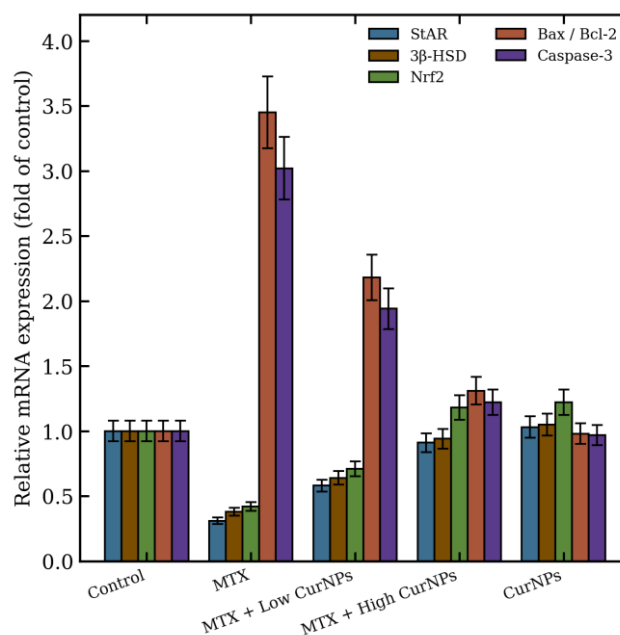


Figure 6. Relative testicular mRNA expression (fold of control) of steroidogenic (StAR, 3 β -HSD), antioxidant (Nrf2) and apoptotic (Bax/Bcl-2, caspase-3) transcripts. β -actin was used as the internal reference.

Table 3. Primer sequences used for quantitative real-time PCR analysis.

Gene	Forward primer (5' $\rightarrow$ 3')	Reverse primer (5' $\rightarrow$ 3')	Product (bp)
StAR	TGTCAAGGAGATCAAGGTC CTG	CGATAGGACCTGGTTGATG AT	168
3 β -HSD	AGGCCTGTGTCCAAGCTAG TGT	CTCGCCATCTTTTGTCTGT AT	152
Nrf2	CACATCCAGACAGACACCA GT	CTACAAATGGGAATGTCTCT GC	194
HO-1	AGGCTTTAAGCTGGTGATG GC	ACGCTTTACGTAGTGCTGTG T	176
Bax	GTGAGCGGCTGCTTGCTCTG	GGTCCGAAGTAGGAGAGG A	161
Bcl-2	GACTGAGTACCTGAACCGG CA	GAGACAGCCAGGAGAAATC AA	143
Caspase-3	GAGCTTGAACGCGAAGA AA	TAACCGGGTGCGGTAGAGT A	150
β -actin	GGAGATTACTGCCCTGGCT CCTA	GACTCATCGTACTCCTGCTT GCTG	150

3.7 Histopathology

Microscopic examination of control sections showed the expected architecture: regular seminiferous tubules with intact germinal epithelium and abundant spermatogenic cells at various stages of maturation. MTX-exposed testes, in contrast, were riddled with empty or partially depleted tubules, vacuolated Sertoli cells, sloughed germ cells and interstitial oedema. Both CurNP doses preserved the general tubular outline, with the high-dose group displaying near-normal histology except for occasional patchy spermatogenic loss. Animals receiving CurNPs alone displayed an essentially intact architecture indistinguishable from controls.

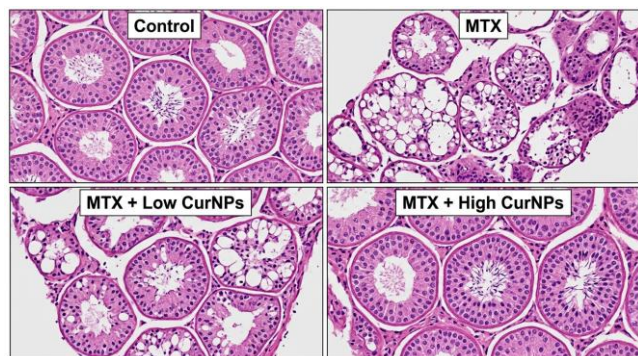


Figure 7. Histopathology comparison panel. Representative H&E-stained testicular sections across experimental groups showing normal seminiferous tubules in the control group, marked degeneration after MTX exposure, partial improvement with low-dose CurNPs, and near-normal restoration with high-dose CurNPs.

4. CONCLUSION

A simple and reproducible green technique based on an aqueous plant extract as dual reducing and capping agent delivered spherical and colloiddally stable curcumin nanoparticles of around 85 nm with an encapsulation effectiveness of about 78 %. These CurNPs, given orally to rats submitted to a toxic dosage of methotrexate, resulted in a clear, dose-dependent restoration of the male reproductive endpoints reported herein. The high-dose regimen, especially, normalised circulating testosterone, LH and FSH; reversed the oxidative imbalance imposed by MTX; recovered sperm count, motility and morphology; and modulated key transcripts of the steroidogenic, antioxidant and apoptotic machineries (StAR, 3 β -HSD, Nrf2, HO-1, Bax/Bcl-2, caspase-3). Histopathological findings showed mostly maintained seminiferous architecture. Although care should be taken before extending these rodent findings to the clinic, the confluence of hormonal, oxidative, molecular and structural evidence points strongly to green-synthesized CurNPs as a viable supplementary method to alleviate the reproductive toxicity that limits MTX therapy. Further pharmacokinetic and long-term safety investigations are necessary to confirm translational potential, preferably in bigger species and across varied MTX dose schedules.

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