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Therapeutic effects of curcumin nanoemulsion on cyclophosphamide-induced testicular toxicity in adult male mice

Pourya Raee[1,2,*], Shahin Aghamiri[3,*], Mahsa Ghaffari Novin[2], Azar Afshar[2], Fakhroddin Aghajanpour[2], Farid Abdi[1], Marefat Ghaffari Novin[1,2]

[1]Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran; [2]Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran; [3]Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Objective: Several chemotherapeutic agents, including cyclophosphamide (CP) and busulfan, have been shown to interfere with spermatogenesis. Accordingly, the main objective of this study was to evaluate the potential therapeutic effects of curcumin nanoemulsion (CUR-NE) on spermatogenesis in mice with CP-induced testicular toxicity.
Methods: A total of 28 adult male mice were equally divided into four groups: control, CUR-NE (30 mg/kg, daily for 5 weeks), CP (200 mg/kg, single dose), and CP+CUR-NE. Each group was evaluated regarding sperm parameters, DNA fragmentation index, chromatin maturation, reactive oxygen species (ROS) levels, and histological parameters of the testes. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone, and testosterone were also assessed in all groups.
Results: In CP-induced mice, CUR-NE treatment significantly improved sperm parameters, including total sperm count, motility, morphology, and DNA integrity. CUR-NE administration was also associated with significantly higher serum levels of testosterone and FSH, as well as testis weight and volume, in the mice treated with CP. Furthermore, CUR-NE treatment significantly increased the number of spermatogonia, primary spermatocytes, round spermatids, and Leydig cells in the testicular tissue of these animals. A marked reduction in ROS levels in the testes tissue was observed following administration of CUR-NE to CP-induced mice.
Conclusion: CUR-NE appears to promote spermatogenesis in mice with CP-induced testicular toxicity by reducing ROS levels, improving testicular stereological parameters, and strengthening the reproductive hormone profile.

Keywords: Curcumin nanoemulsion; Cyclophosphamide; Reactive oxygen species; Spermatogenesis; Testis

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Corresponding author: **Pourya Raee**
Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Daneshjoo Blvd, Velenjak, Shahid Chamran Highway, Tehran, Iran
Tel: +98-2123872555 Fax: +98-2122439976 E-mail: pourayarayi3@gmail.com

Co-corresponding author: **Marefat Ghaffari Novin**
Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Daneshjoo Blvd, Velenjak, Shahid Chamran Highway, Tehran, Iran
Tel: +98-2123872555 Fax: +98-2122439976 E-mail: mghaffarin@yahoo.com

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Introduction

Chemotherapy is often considered the primary treatment modality for cancer. However, exposure to chemotherapeutic agents can adversely affect spermatogenesis and damage sensitive germ cells. Cells that proliferate rapidly, such as spermatogenic cells, are particularly susceptible to the gonadotoxic effects of chemotherapy [1,2]. Consequently, many cancer treatments are associated with a reduction in fertility. Chemotherapeutic drugs have the potential to cross the blood-testis barrier, thereby posing a risk to testicular tissue through various harmful effects [1,2]. The efficacy of these treatments—particularly alkylating agents—depends on their mechanism of action, with outcomes varying according to the dosage and

duration of the treatment [1,2].

Cyclophosphamide (CP), an alkylating agent, is widely used as an anticancer drug and immunosuppressant. It is a staple in clinical practice for treating malignant leukemias, lymphomas, ovarian carcinoma, and breast cancer, often administered alongside other chemotherapy medications [3,4]. Studies have demonstrated that CP treatment significantly reduces testicular weight and induces histological abnormalities, which are key indicators of reproductive toxicity [3,5]. This chemotherapeutic agent is linked to a marked decrease in sperm concentration, motility, and morphology. The underlying mechanisms involve DNA damage, oxidative stress, and inhibition of enzymes associated with the tricarboxylic acid cycle, leading to a reduction in available adenosine triphosphate (ATP). Additionally, exposure to reactive oxygen species (ROS) results in lower ATP levels, which in turn impairs sperm motility [3,6]. Treatment with CP leads to reduced serum concentrations of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), as well as diminished activities of testicular steroidogenic enzymes, including cytochrome P450scc, aromatase, and 17-hydroxysteroid dehydrogenase. These effects may stem from CP-induced oxidative stress and changes in gene expression patterns in Leydig cells [3,7]. Moreover, phosphoramide mustard, a metabolite of CP, can inhibit DNA replication by promoting cross-linking within the DNA strands. This significantly reduces cell division through mitosis and meiosis, adversely impacting spermatogenesis [3].

The potential therapeutic use of various antioxidants to mitigate the adverse effects of chemotherapy on the male reproductive system has been proposed [8-11]. Curcumin is one such antioxidant that may be employed for this purpose. This naturally derived compound is extracted from the rhizome of *Curcuma longa*, a member of the Zingiberaceae family [12,13]. The therapeutic and biological properties of curcumin have established its prominence in traditional medicine. Its therapeutic effects can be ascribed to three biological functions: antioxidant action, protein binding capacity, and metal-chelating activity [12,13]. Additionally, curcumin possesses several other beneficial properties, such as preserving mitochondrial function, modulating proinflammatory transcription factors, inhibiting apoptosis, and reducing inflammation. These characteristics are highly relevant in the management of a variety of health conditions [12,13]. Consequently, curcumin is an excellent candidate for reducing the side effects of cancer therapy, particularly reproductive toxicity. Both alone and in combination with other protective agents, curcumin has demonstrated efficacy in mitigating chemotherapy-induced testicular damage [14-17].

It is well-established that the aromatic groups in the structure of curcumin contribute to its hydrophobic nature, while the α, β-unsaturated β-diketo moiety of the molecule imparts flexibility. These distinctive features enable curcumin to effectively interact with a diverse range of biomacromolecules, including nanoemulsions (NEs) [13]. Thus, novel nano-drug formulation and delivery approaches can potentially augment the therapeutic benefits of curcumin. One such approach involves the use of NE-based nanocarriers as drug delivery vehicles. NEs are emulsions with nanoscale dimensions ranging from tens to hundreds of nanometers. These nano-sized emulsions offer several appealing attributes: small particle sizes, a large surface area relative to volume, improved dispersion of hydrophobic components, and higher absorption capabilities [18]. Additionally, NEs are biologically safe and significantly increase the bioavailability of hydrophobic (lipophilic) bioactive substances [19]. Therefore, NEs are promising nanocarriers for curcumin, with the added advantage of increasing the compound's bioavailability without compromising its biological safety.

Hence, the NE-based formulation of curcumin shows promise as a treatment option for mitigating testicular damage induced by CP. Nevertheless, no data are yet available on the potential impact of curcumin nanoemulsion (CUR-NE) treatment on CP-induced testicular toxicity in mice.

Methods

1. Preparation and characterization of CUR-NE

For the preparation of CUR-NE, soybean oil was utilized for the oily phase, Tween 80 and Tween 85 served as surfactants, and ethanol acted as the co-surfactant. The CUR-NE was formed using a spontaneous emulsification procedure [20,21]. The particle size distribution of CUR-NE was determined using dynamic light scattering with a Scatteroscope device (K-One Co. Ltd.). The zeta potential of CUR-NE was assessed with a zeta potential analyzer (SZ-100; HORIBA).

2. Animals and experimental design

This study was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.AEC.1401.027) and was conducted in strict accordance with the relevant guidelines and regulations. A total of 28 adult male NMRI mice, 8 weeks old and weighing 25–30 g, were housed in a controlled environment at room temperature.

The mice were kept under a 12-hour light/dark cycle and were given *ad libitum* access to food and water. The animals were randomly assigned into four groups, with seven mice in each group: (1) control, (2) CUR-NE (30 mg/kg, administered intraperitoneally [ip] daily for 5 weeks), (3) CP (200 mg/kg, ip, single dose), and (4) a combination of CP+CUR-NE. For subsequent analyses, the mice were sacrificed using a ketamine/xylazine combination (80 and 10 mg/kg, respectively, ip). Figure 1 illustrates the detailed timeline of the study procedures. At

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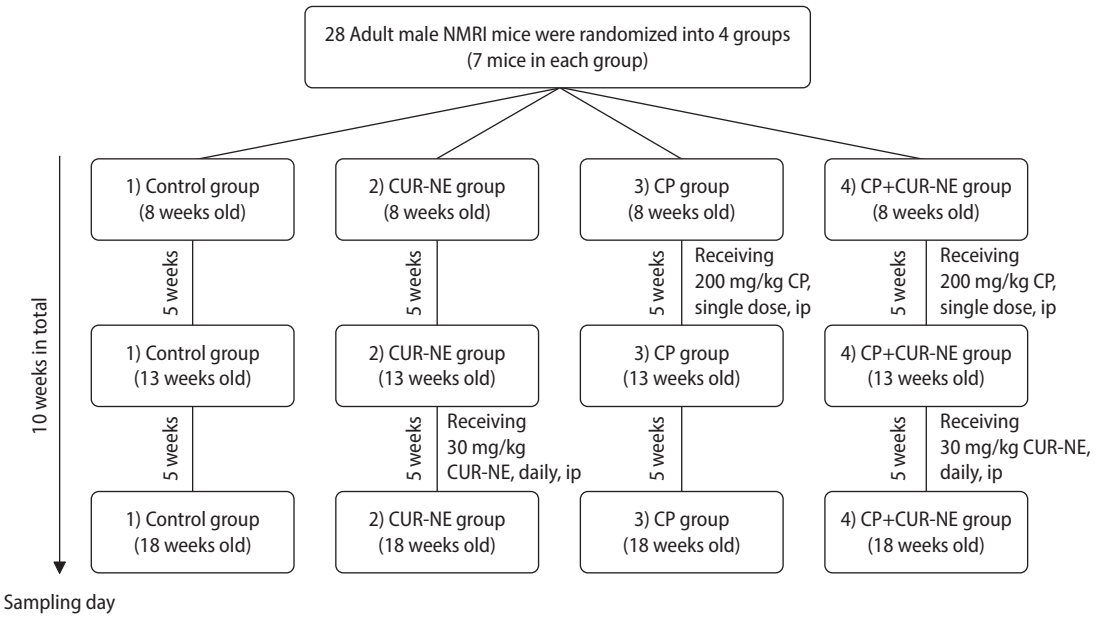

Figure 1. Detailed timeline of the present study. CUR-NE, curcumin nanoemulsion; CP, cyclophosphamide; ip, intraperitoneal.

the time of sacrifice, all mice across the groups were the same age (18 weeks old).

3. Semen analysis

The caudal region of the epididymis was excised and placed in a Petri dish containing 1 mL of pre-warmed Ham F10 medium. It was then minced and incubated at 37 °C with 5% carbon dioxide for 15–20 minutes. Following incubation, 10 µL of the sperm mixture was used to evaluate sperm parameters. Sperm count, motility, and morphology were assessed using a bright-field microscope, following the previously described method [22]. The DNA fragmentation index (DFI) was assessed using the sperm chromatin dispersion method with a sperm DNA fragmentation assay kit (IVF Co.), in accordance with the manufacturer's instructions. The percentage of sperm exhibiting DNA fragmentation was then calculated [23]. Sperm chromatin maturation was evaluated using an aniline blue staining kit (IVF Co.), again following the manufacturer's instructions. The proportion of sperm with immature chromatin, indicated by blue-stained nuclei, was subsequently reported [24,25].

4. Tissue preparation and stereological study

The tissue samples were fixed in Bouin solution for 24 hours. Serial sections of 5 and 20 µm thicknesses were obtained using a rotary microtome. These sections were then stained with hematoxylin and eosin. Sections for analysis were selected using systematic uniform random sampling. Stereological studies were performed in a blinded manner. The total volume of the testis was estimated using the point counting method in conjunction with the Cavalieri principle, based on the following formula [22]:

$$V = \sum P \times \frac{a}{p} \times t$$

Here, ΣP represents the number of points counted. *a/p* indicates the area of the probe points divided by the magnification, and *t* denotes the distance between tissue sections.

The optical dissector technique was employed to estimate the numerical density (*Nv*) of cells within the testes, using the following equation [22]:

$$N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

Here, ΣQ represents the estimated number of testicular cells. This estimate was obtained using a counting frame probe and a microcator (Heidenhain) attached to the microscope stage. The microcator measured the height of the dissector (*h*) and the actual thickness of the tissue section (*t*). Next, ΣP denotes the total number of counted fields. The term *a/f* refers to the ratio of the probe's area to the magnification, while *BA* indicates the thickness of the tissue sections.

5. Measurement of ROS levels

After the isolation of testicular cells using trypsin-ethylene-diamine-tetraacetic acid (EDTA), these samples were suspended in phosphate-buffered saline and centrifuged at 1,200 revolutions per minute and 4 °C for 5 minutes. Subsequently, 20 µM of dichlorodihy-

drofluorescein diacetate (DCFDA) was introduced to the sample as a 100-μL aliquot, followed by incubation at 37 ℃ for 45 minutes. Flow cytometry was then applied for analysis [26].

6. Hormonal assessment

Blood samples were collected via cardiac puncture while under deep anesthesia. These samples were then allowed to clot at 37 ℃ for 1 hour. Afterward, the clot was cooled to 4 ℃ for 2 hours to promote contraction. The serum was subsequently separated from the clot and centrifuged at 10,000 ×g for 10 minutes at 4 ℃ to remove any remaining insoluble particles. The serum samples were stored at −80 ℃ until further analysis [27]. Mouse-specific enzyme-linked immunosorbent assay kits were used to assess serum levels of FSH (BT Lab), LH, and testosterone (Novus Biologicals).

7. Statistical analysis

Analyses were conducted using GraphPad Prism ver. 9 (GraphPad Software Inc.). Data are presented as mean±standard deviation. The Shapiro-Wilk test was used to assess the normality of the distribution, and significance was determined by one-way analysis of variance followed by the Tukey *post hoc* test. *p*-values of less than 0.05 were considered to indicate statistical significance.

Results

1. Characterization of the CUR-NE

As presented in Figure 2, the CUR-NE was measured at 13.8 nm, and the zeta potential was found to be −10.6 mV (Figure 2).

2. Sperm parameters

As shown in Figure 3, the administration of CP significantly reduced sperm count, total motility, progressive motility, normal morphology, and sperm DNA integrity (as evidenced by increased DFI levels) in the CP group compared to both the control and CUR-NE

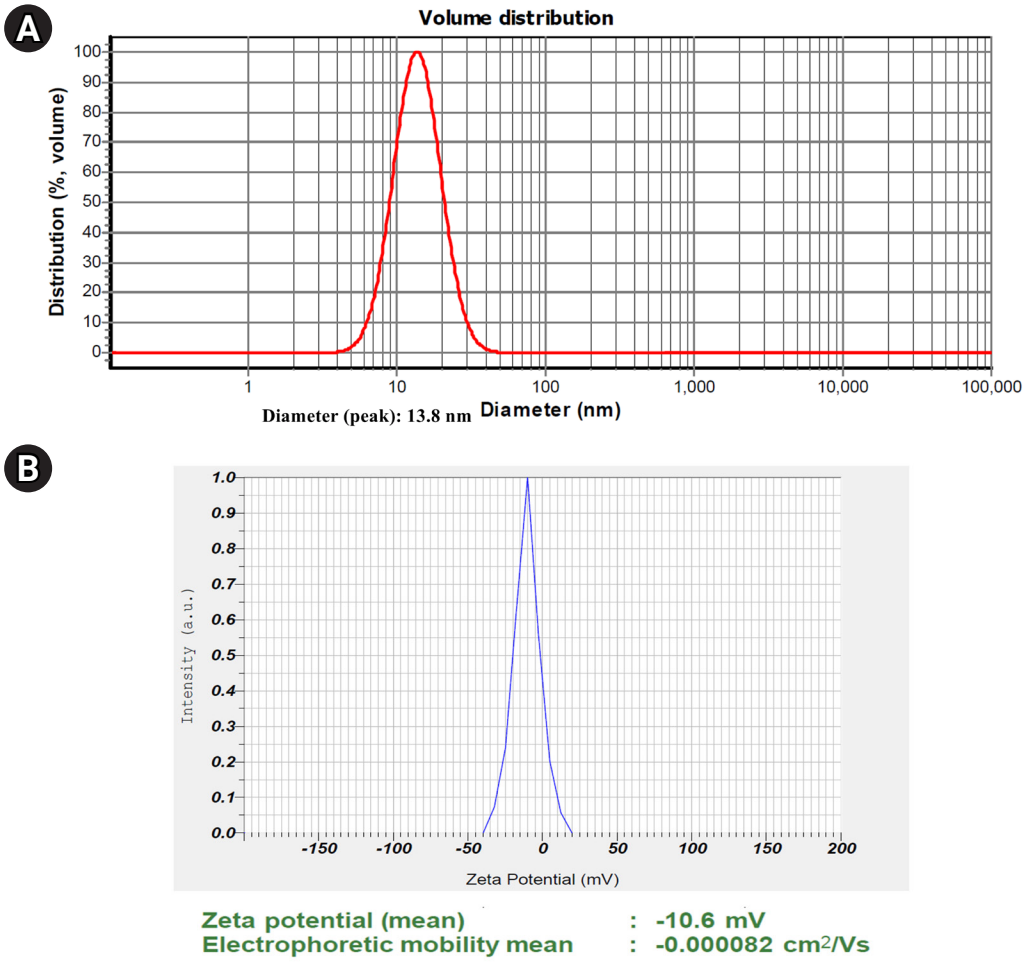

Figure 2. Characterization of the curcumin nanoemulsion. (A) Size distribution as determined by dynamic light scattering. (B) Zeta potential as measured using a zeta potential analyzer.

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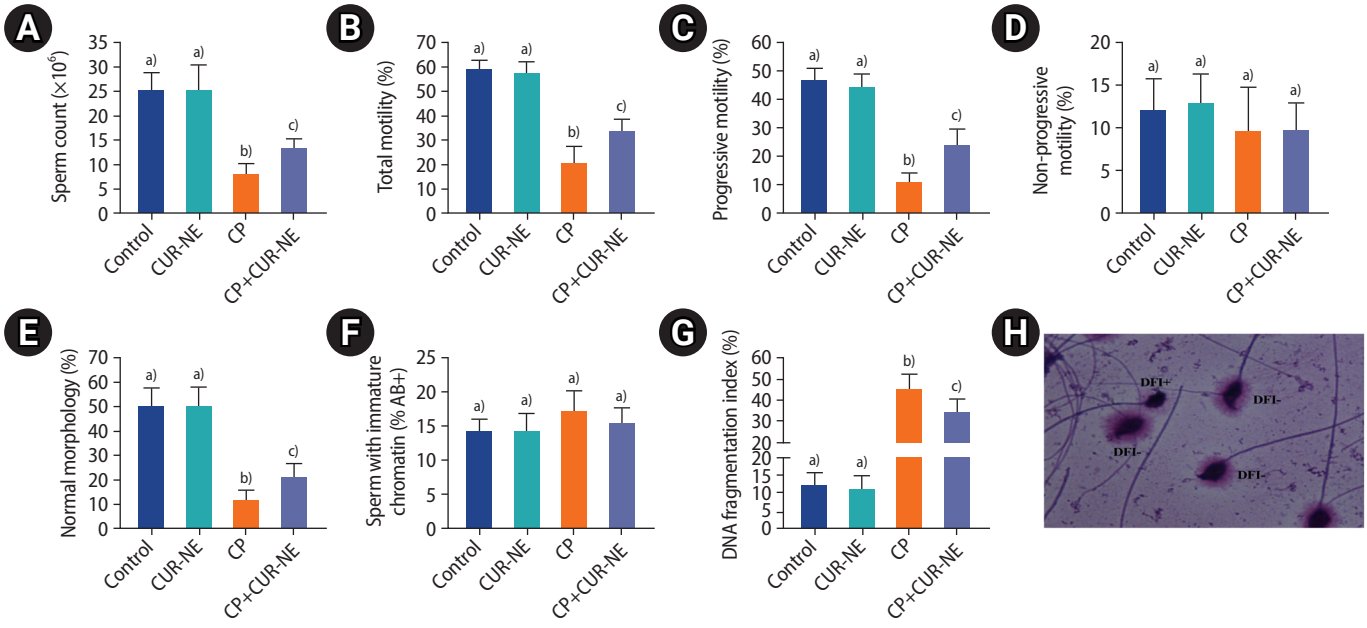

Figure 3. Comparison of sperm parameters between study groups. (A-G) All data are presented as mean±standard deviation. (H) Photomicrograph of DNA fragmentation index (DFI)+ and DFI– sperm under ×100 magnification. CUR-NE, curcumin nanoemulsion; CP, cyclophosphamide; AB, anilin blue. [a),b),c)]No significant difference among the groups ($p>0.05$), while distinct letters denote a significant difference (specifically at $p<0.05$).

groups ($p<0.0001$). Treatment with CUR-NE significantly improved sperm count ($p<0.05$), total motility ($p<0.001$), progressive motility ($p<0.001$), normal morphology ($p<0.05$), and sperm DNA integrity ($p<0.01$) in the CP+CUR-NE group relative to the CP group. However, despite these improvements, these parameters remained significantly lower in the CP+CUR-NE group compared to the control and CUR-NE groups ($p<0.0001$).

No significant differences were observed between the study groups in terms of sperm non-progressive motility and chromatin maturation ($p>0.05$) (Figure 3D, 3F). Furthermore, no significant differences were found in any of the mentioned parameters between the control and CUR-NE groups ($p>0.05$) (Figure 3A-3G).

3. Stereological parameters

1) Testicular volume and weight

It should be noted that the photomicrograph showing seminiferous tubes stained with hematoxylin and eosin in the studied groups is presented in Figure 4. As indicated in Figure 5A, 5B, CP injection resulted in a significant reduction in both the weight and volume of testicular tissue in the CP group compared to the control and CUR-NE groups ($p<0.0001$). Additionally, treatment of CP-induced mice with CUR-NE significantly increased (that is, improved) testicular weight ($p<0.01$) and volume ($p<0.05$) compared to the CP group. Nevertheless, the testicular weight and volume in the CP+CUR-NE group remained significantly lower than those in both the control and CUR-NE groups ($p<0.01$). No significant differences in these parameters were identified between control and CUR-NE groups ($p>0.05$).

2) Cell counts in testicular tissue

As indicated in Figure 5C-5E, and 5G, CP injection resulted in a significant reduction in the number of spermatogonia, primary spermatocytes, spermatids, and Leydig cells in the testicular tissue of the CP group compared to both control and CUR-NE groups ($p<0.0001$). Treatment with CUR-NE significantly increased the numbers of spermatogonia ($p<0.05$), primary spermatocytes ($p<0.05$), spermatids ($p<0.05$), and Leydig cells ($p<0.001$) in the testicular tissue of the treatment group (CP+CUR-NE) relative to the CP group. As presented in Figure 5F, no significant difference between groups was observed in the number of Sertoli cells ($p>0.05$). Furthermore, no significant differences were identified between the control and CUR-NE groups in the above parameters ($p>0.05$). The photomicrograph showing an example of each cell type in hematoxylin and eosin stained testes tissue is presented in Figure 5H.

4. ROS levels

The results presented in Figure 6A indicate a significant increase in ROS level within the testicular tissue of the CP group compared to both the control and CUR-NE groups ($p<0.0001$). Treatment with CUR-NE resulted in a significant decrease in this parameter in the

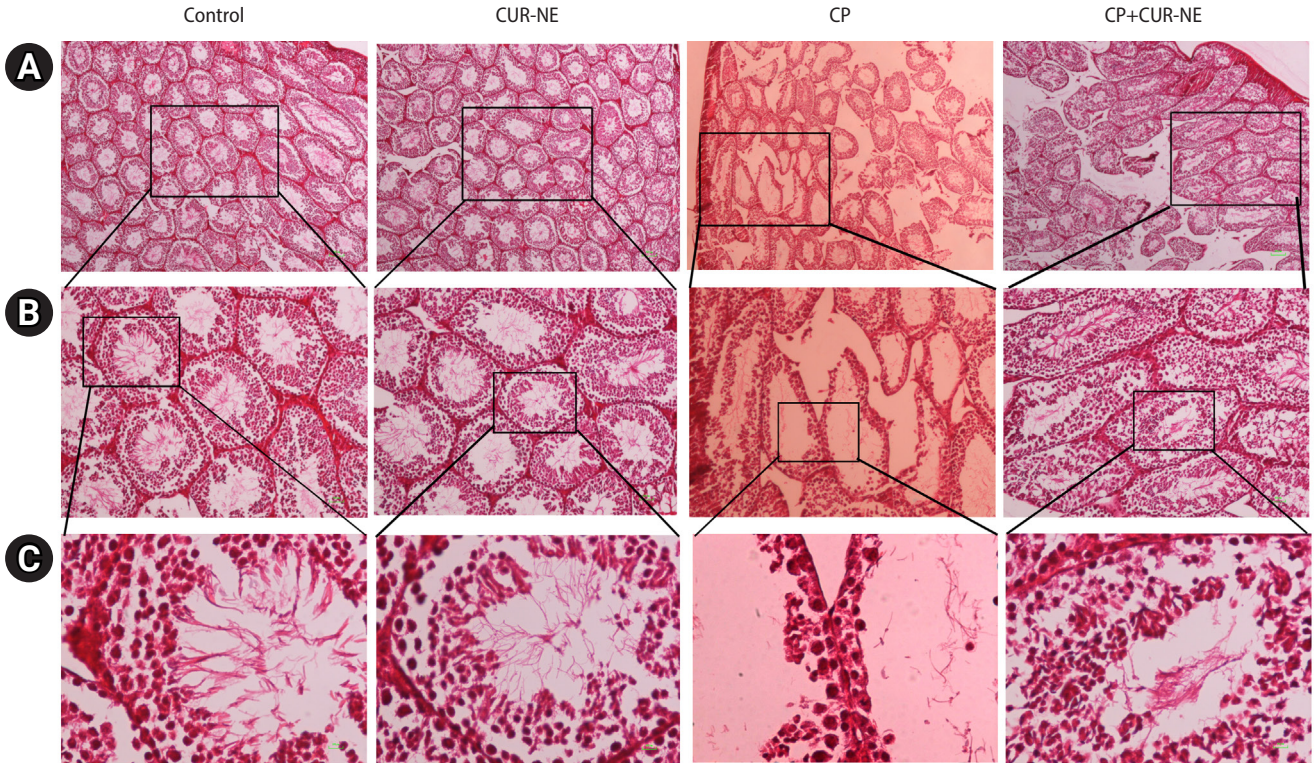

Figure 4. Photomicrograph showing hematoxylin and eosin stained seminiferous tubules in the study groups, captured at magnifications of (A) ×4, (B) ×10, and (C) ×40. CUR-NE, curcumin nanoemulsion; CP, cyclophosphamide.

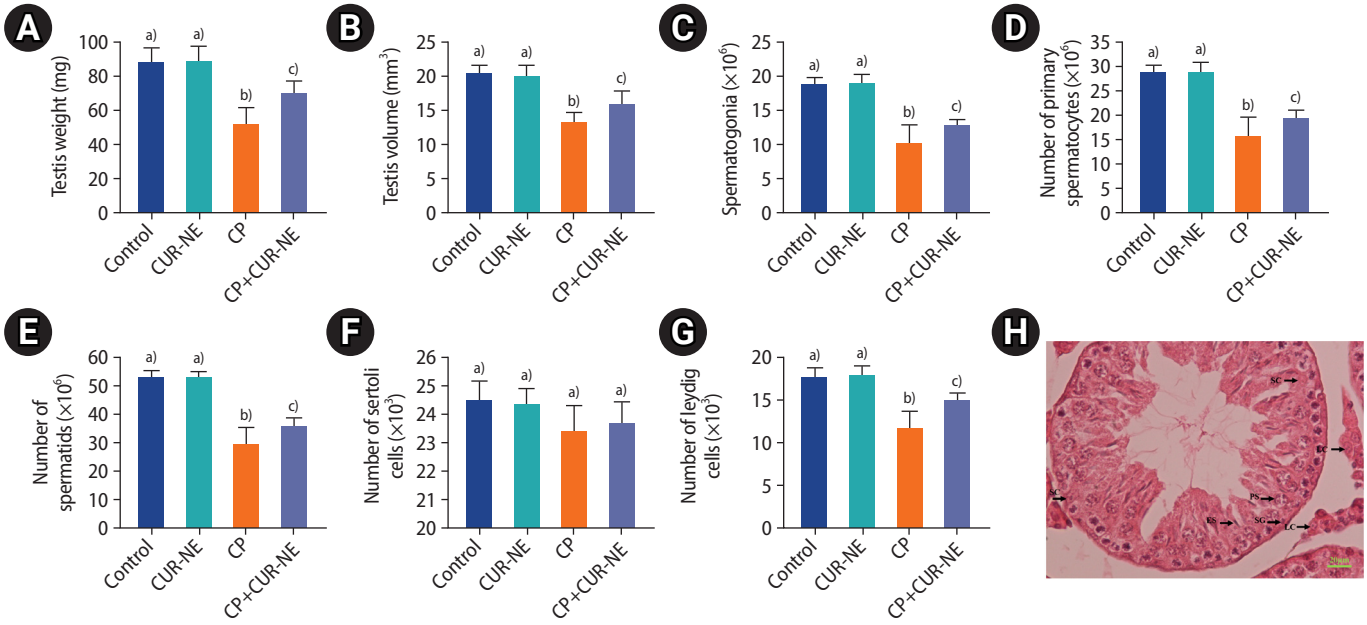

Figure 5. Comparison of testis weight, volume, and numbers of testicular cell types between study groups. (A-G) All data are presented as mean±standard deviation. (H) Photomicrograph showing an example of each cell type in hematoxylin and eosin stained testes tissue (×40 objective). CP, cyclophosphamide; CUR-NE, curcumin nanoemulsion; LC, Leydig cell; SC, Sertoli cell; SG, spermatogonia; PS, primary spermatocyte; ES, elongated spermatid. [a),b),c)]No significant difference among the groups ($p>0.05$), while distinct letters denote a significant difference (specifically at $p<0.05$).

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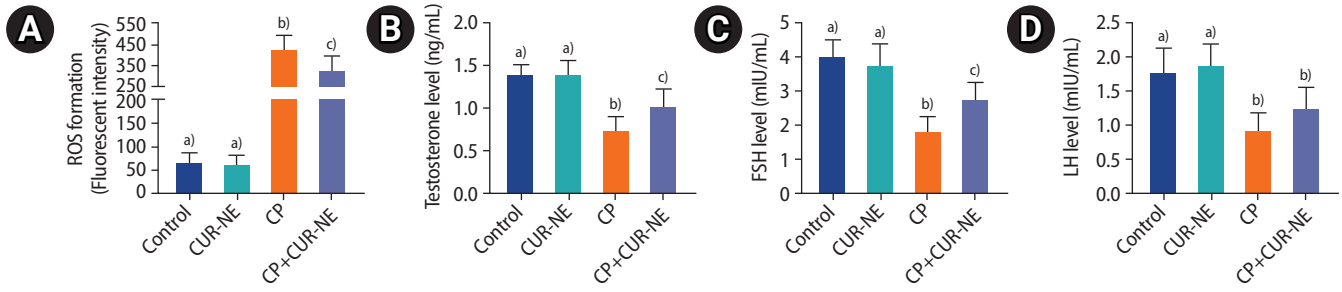

Figure 6. Comparison of (A) reactive oxygen species (ROS), (B) testosterone, (C) follicle-stimulating hormone (FSH), and (D) luteinizing hormone (LH) levels between study groups. All data are presented as mean±standard deviation. CUR-NE, curcumin nanoemulsion; CP, cyclophosphamide. [a),b),c)]No significant difference among the groups (p>0.05), while distinct letters denote a significant difference (specifically at p<0.05).

mice treated with CP+CUR-NE relative to the CP animals (p<0.01). Despite this reduction, ROS levels in the testicular tissue of the CP+CUR-NE group remained significantly elevated compared to the control and CUR-NE groups (p<0.0001). Furthermore, no significant difference in ROS levels was observed between the control and CUR-NE groups (p>0.05).

5. Hormonal assessment

The findings (Figure 6B-6D) indicate significantly lower serum testosterone, FSH, and LH levels in the CP group compared to the control (p<0.0001, p<0.0001, and p<0.001, respectively) and CUR-NE (p<0.0001 for all) groups. Treatment with CUR-NE led to significant increases in testosterone and FSH levels in the CP+CUR-NE animals relative to the CP group (p<0.05). However, testosterone and FSH concentrations remained significantly lower in the CP+CUR-NE group compared to the control and CUR-NE groups (p<0.01). Additionally, the CP+CUR-NE group exhibited an increase in serum LH levels compared to the CP group, but this finding lacked statistical significance (p>0.05). No significant differences in any of these parameters was detected between the control and CUR-NE animals (p>0.05).

Discussion

Our investigation into the therapeutic effects of CUR-NE in a mouse model of CP-induced testicular toxicity revealed multifaceted insights into potential mechanisms, therapeutic significance, and avenues for future exploration. With few exceptions, prior studies exploring the protective/therapeutic effects of antioxidants on CP-induced testicular toxicity have focused on raw agents; in contrast, our utilization of CUR-NE represents a novel approach that optimizes bioavailability.

The significant decreases in sperm parameters induced by CP underscore the need for protective interventions. CUR-NE demonstrated promising efficacy in ameliorating sperm count, motility, morphology, and DNA integrity in CP-induced mice. Additionally, CUR-NE treatment significantly increased testicular weight, volume, and cell numbers, indicating its potential to counteract the adverse effects of chemotherapy on testicular tissue. These results align with other studies that have employed curcumin to address testicular damage caused by various stressors, including different chemotherapeutic agents (cisplatin and doxorubicin) and scrotal hyperthermia [16,17,28].

Several studies have investigated the protective and therapeutic effects of curcumin on models of testicular toxicity induced by CP [15,29]. However, these display notable differences in study design and curcumin formulation compared with the present study. One study administered curcumin as a protective agent against CP-induced testicular toxicity in a rat model. In that study, the post-treatment group received curcumin (without any nano-drug delivery system) for 14 consecutive days following a single injected dose of CP (150 mg/kg) [29]. The findings indicated that curcumin improved sperm quality and quantity, while also reducing inflammation and caspase-3 activity [29]. This study's design diverges from ours, as we first established a CP-induced testicular toxicity model in mice (200 mg/kg, single dose) and then waited 5 weeks (to cover one spermatogenesis cycle) before starting treatment with CUR-NE using NEs as a drug delivery system. Another study explored the use of curcumin nanocrystals for treating CP-induced testicular toxicity [15]. The researchers observed improvements in the architecture of the seminiferous tubules, as well as sperm chromatin condensation and quality. The study successfully applied nanotechnology to treat CP-induced testicular toxicity with curcumin nanocrystals [15]. In contrast, our research employed NEs as a nano-drug delivery system to potentially further augment the therapeutic effects of curcumin.

In this study, the observed improvements in sperm and testicular histological parameters in a CP-induced mouse model following treatment with CUR-NE align with curcumin's known antioxidant

and anti-inflammatory properties. Research has demonstrated that the nuclear factor kappa B (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK) signaling pathways play crucial roles in the development of CP-induced testicular toxicity in animal models, with both pathways actively involved in inflammatory responses [3,30]. Notably, curcumin has been shown to exert its effects through the inhibition of NF-κB and MAPK pathways [31,32]. Further studies on various animal models of testicular toxicity/injury have revealed that curcumin can mitigate testicular damage by inhibiting NF-κB and p38 MAPK cascades, leveraging its anti-inflammatory and anti-apoptotic properties [33,34]. Additionally, a report suggests that the immunomodulatory effects of curcumin in cases of testicular toxicity can be significantly increased through nanoparticle-based delivery [35].

Nevertheless, the potential roles of key signaling pathways, such as NF-κB and p38 MAPK, warrant additional investigation to elucidate the precise molecular mechanisms by which CUR-NE exerts its therapeutic effects on spermatogenesis in the context of CP-induced testicular toxicity.

The observed increase in ROS levels following CP treatment highlights oxidative stress as an important contributor to reproductive toxicity. CP administration reduces the level of nuclear factor erythroid 2–related factor 2 (Nrf2), which is a crucial regulator of antioxidant response element (ARE)–dependent genes. These genes encode multiple proteins that assist in cellular adaptation and survival under oxidative stress [3,36]. Additionally, a deficiency in Nrf2 renders cells more vulnerable to oxidants [3,36]. In the present study, CUR-NE decreased ROS levels in CP-induced testicular toxicity, which is consistent with its known antioxidant properties and increased bioavailability. Previous research has demonstrated that curcumin administration can effectively activate the Nrf2 pathway, thereby reducing oxidative stress in various models of testicular toxicity [37-39]. Furthermore, using an optimized NE-based delivery system for curcumin can significantly amplify its antioxidant effects due to increased solubility, bioavailability, and improved intracellular uptake and cell internalization [40,41]. The precise mechanisms by which CUR-NE modulates oxidative stress, including the Nrf2/ARE pathway, require further investigation to fully elucidate its antioxidant actions.

CP-induced hormonal imbalances, characterized by decreased levels of testosterone, FSH, and LH, highlight the endocrine disruption associated with chemotherapy. CP has been demonstrated to compromise male fertility by reducing the activity of the hypothalamic–pituitary–gonadal (HPG) axis [42]. This is achieved by reducing the secretion of gonadotropins, disrupting testicular steroidogenesis, inhibiting the synthesis of testosterone, and consequently impairing spermatogenesis [42]. Treatment with CUR-NE resulted in a marked increase in testosterone and FSH levels, indicating its potential to modulate the endocrine milieu. While a prior study demonstrated the protective effect of curcumin on the HPG axis in the context of CP-induced testicular toxicity [29], no previous reports have covered CUR-NE formulation. Further research into the interactions between CUR-NE and hormonal signaling pathways, such as the HPG axis, is crucial to fully understand the hormonal effects of this treatment.

In the present study, we observed no significant difference in the study parameters between the control and CUR-NE–treated groups. This suggests that administering CUR-NE (30 mg/kg ip, daily for 5 weeks) does not adversely affect spermatogenesis or related parameters in healthy adult male mice.

The observed improvements in sperm parameters, hormonal profile, and testicular histology underscore the therapeutic potential of CUR-NE in alleviating chemotherapy-induced reproductive toxicity. Future research should examine the long-term impacts of CUR-NE, its underlying molecular mechanisms, potential combination strategies with other therapies, and its applicability across diverse preclinical and clinical settings.

In conclusion, our study clarifies the potential of CUR-NE to mitigate CP-induced testicular toxicity. The findings suggest that CUR-NE improves spermatogenesis in a CP-induced mouse model by reducing ROS levels, improving testicular histology, and strengthening the reproductive hormone profile.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

ORCID

Pourya Raee https://orcid.org/0000-0001-7262-9658
Shahin Aghamiri https://orcid.org/0000-0003-1083-1409
Marefat Ghaffari Novin https://orcid.org/0000-0003-4455-5211

Author contributions

Conceptualization: PR, MGN. Methodology: PR, SA, MGN, AA, FA, FA. Formal analysis: PR. Data curation: PR, SA, MGN, AA, FA, FA. Funding acquisition: MGN. Project administration: MGN. Visualization: PR. Software: PR, SA. Validation: MGN. Investigation: PR. Writing-original draft: PR, SA. Writing-review & editing: PR, SA, MGN. Approval of final manuscrip: PR, SA, MGN, AA, FA, FA, MGN.

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