# Querectin Alleviates Zinc Oxide Nanoreprotoxicity in Male Albino Rats

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**ABSTRACT: Zinc oxide nanopartciles (ZnONPs) involved in advanced technologies, and their wide-scale use in consumer market makes human beings more prone to the exposure to ZnONPs. The present study was undertaken to evaluate amelioration of ZnONP-induced toxicities with querectin in male albino rats. ZnONPs-treated rats showed a significant decrease in sperm cell count, sperm motility, live and normal sperms, as well as serum testosterone level. Severe histopathological damage with a significant increase in lipid peroxidation and a decrease in antioxidant enzymes activity and the GSH level were observed in the affected testis. Relative quantitative polymerase chain reaction results showed a significant decrease in antioxidant enzymes (superoxide dismutase and catalase) and a significant decrease in 3$\beta$-HSD, 17$\beta$-HSD, and Nr5A1 transcripts. Rats-administered querectin along with ZnONPs showed less toxic effects on all studied reproductive traits and mRNA transcripts. Our results suggest that querectin is beneficial for preventing or ameliorating ZnONP reproductive toxicities in males.**
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KEYWORDS: **Zinc Oxide Nanoparticles; Querectin; Male Rats; Sperm; Antioxidants**

## INTRODUCTION

Nanotechnology has helped in studying and developing materials with surface structure and chemical properties on the nanoscale dimension and with special properties arising from this. One of the definitions of the term nanoscale is particles that are <100 nm in at least one dimension. Nanotechnology has rapidly emerging industrial applications in medical, industrial, and military areas [1, 2]. Zinc oxide nanoparticles (ZnONPs) can be found in a variety of sources including semiconductors, catalysts, and paints as well as food industry, cosmetic products, and sunscreen lotions, because of the strong UV absorption properties of ZnO [3]; however, its accumulation in the tissues raises the question as to whether ZnONPs cause toxic or harmful disturbances in human health, especially on the reproductive system, and remains a matter of concern. Few studies showed the reproductive toxicity of ZnONP both in vivo [4] and in vitro conditions [5].

In vivo studies owed to the main mechanism of ZnONP toxicities due to the oxidative stress observed after ZnONP treatment in animal models [6–9].

Oxidative stress has been recognized as one of the most important cause of male infertility because free radicals and reactive oxygen species can damage sperm function and DNA integrity [10]. Since antioxidants suppress the action of reactive oxygen species, these compounds have been used in the medical treatment of male infertility or have been added to the culture medium during sperm separation techniques [11].

Querectin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one] is a plant-derived flavonoid, which is mainly found in fruits and vegetables. Several studies have indicated that querectin may have anti-inflammatory and antioxidant properties due to its free radical scavenging and metal-chelating activities [12–14].

Therefore, the present study was undertaken to study the nanoreprotoxicity of ZnONP administration

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in male albino rats and the effect of querectin supplementation on these affected rats.

## MATERIALS AND METHODS

### Chemicals

Zinc oxide nano powder [(ZnO), CAS registry number 1314-13-2, <50 nm average particle size (APS), purity >97% with surface area >10.8 $m^2/g$], querectin [($C_{15}H_{10}O_{7)}$, CAS registry number 117-39-5, purity ≥95%], epinephrine, DTNB (5,5 dithiobis 2-nitrobenzoic acid), and NADPH were purchased from Sigma Aldrich(St. Louis, MO). Potassium dichromate, hydrogen peroxide, glacial acetic acid, and EDTA were purchased from El-Nasr (Cairo, Egypt). A radioimmunoassay kit for serum testosterone (TESTO-CTK) was obtained from Dia-Sorin (Stillwater, MN).

### Animal Management

Sixty healthy adult male albino rats, weighing 100–160 g, were obtained from the Animal House at the Faculty of Veterinary Medicine, Zagazig University, Sharkia, Egypt. Rats were given free access to food and water with a 12 h/12 h dark/light cycle. All animals were left for 15 days for adaptation in standard cages under controlled conditions with free access to food and water. The experiment was conducted following the guidelines and ethical rules of Faculty of Veterinary Medicine, Zagazig University, Egypt.

### Experimental Design

After the acclimation period, rats were randomly segregated into six groups (10 rats per group). Group 1 (control group) rats received normal saline for 12 weeks. Group 2 (querectin-treated group) rats received 100 mg/kg body weight querectin orally once daily for 12 weeks dissolved in distilled water, and this oral protective dose of querectin has been reported previously as a good protective daily dose in rats [15]. Group 3 (low ZnO-treated group) rats received 100 mg/kg body weight ZnO per day for 12 weeks orally dissolved in distilled water, meanwhile group 4 rats received high ZnO-treated group (400 mg/kg body weight) once daily for 12 weeks [16, 17]. Group 5 rats were treated with querectin and low ZnO dose as in groups and 3, whereas group 6 rats were treated with querectin and high ZnO dose as in groups 2 and 4. All treatments were given daily using intragastric intubation.

### Sampling

At the end of the experimental period, rats from all groups were fasted overnight, and blood samples were collected into chilled nonheparinized tubes and centrifuged at 860×*g* for 20 min at 4°C. The separated sera were frozen at –20°C for testosterone analysis. After the collection of blood samples, the animals were sacrificed. Portions from the tests tissue were accurately weighed and homogenized using a tissue homogenizer (Potter–Elvehjem) with chilled potassium chloride (1.17%) for the measurement of antioxidant changes. Another portion of the testes samples was collected in a liquid nitrogen container until the time of RNA extraction to follow up the changes in transcriptional levels of antioxidant enzymes and steroidogenic genes. Meanwhile, small parts of the testis tissues were fixed in neutral formalin solution for histopathological examination.

### Biochemical Analysis

Testes samples were homogenized and examined for oxidative status and antioxidant enzymes according to our previous report [18]. Briefly, testis samples were homogenized by a tissue homogenizer followed by centrifugation to obtain the postmitochondrial supernatant, which was used to assay the malondialdehyde (MDA) lipid peroxidation marker, catalase (CAT) (EC 1.11.1.6), superoxide dismutase (SOD) (EC 1.15.1.1), glutathione peroxidase (GPx) (EC 1.11.1.9), and reduced glutathione (GSH). The level of MDA in testes homogenates was determined spectrophotometrically according to the method of Nair and Turner [19]. GSH levels were determined according to the method of Beutler [20]. CAT activity in the testes was determined spectrophotometrically at a wavelength of 570 nm according to the method of Sinha [21]. The activity of SOD in the testis tissues was determined spectrophotometrically at a wavelength of 480 nm by the epinephrine method and is expressed as units of enzyme [22]. GPx activity was determined by the method of Paglia and Valentine [23]. The optical densities of the given parameters were measured using a Shimadzu-type spectrophotometer (UV 120-02). The serum testosterone levels were estimated using the radioimmunoassay kit with sensitivity of 0.05 ng/mL.

### Epididymal Sperm Analysis

The epididymal sperm analysis was done according to our previous report [24]. In brief, the cauda epididymis of one testis was excised and placed in a sterilized Petri dish containing 2 mL warm normal saline

**TABLE 1.** Primers Used for Relative Quantitative Polymerase Chain Reaction

| *Gene* | | *Oligonucleotide Sequences (5′—3′)* | *Accession No.* |
|---|---|---|---|
| CAT | F | GCGAATGGAGAGGCAGTGTAC | AH004967 |
| | R | GAGTGACGTTGTCTTCATTAGCACTG | |
| CU-ZN, SOD | F | GCAGAAGGCAAGCGGTGAAC | X05634 |
| | R | TAGCAGGACAGCAGATGAGT | |
| 3$\beta$-HSD | F | GCATTAACCCCACTCCCACT | NM_017265 |
| | R | GGACCCTGACCTCCTTCAGA | |
| 17$\beta$-HSD | F | GTGTGCACATTTTCCAAGGC | NM_054007 |
| | R | TTTAACAAACTCATCGGCGG | |
| Nr5A1 | F | CGCCAGGAGTTTGTCTGTCT | NM_001191099 |
| | R | ACCTCCACCAGGCACAATAG | |
| $\beta$-actin | F | CCTGCTTGCTGATCCACA | V01217 |
| | R | CTGACCGAGCGTGGCTAC | |

of 37°C, and then macerated to obtain the epididymal contents in a suspension that was handled exactly as the semen [25]. A drop of the suspension was put on a clean glass slide prewarmed to 37°C and covered by a glass cover slide, also prewarmed to 37°C and examined under high power (40×) of light microscope for evaluating individual motility of the spermatozoa. Ten microscopic fields were examined to evaluate the percentage of motile spermatozoa. One drop of the suspension was put on a glass slide and stained with eosin-nigrosine to determine the ratio of live/dead sperms. For the sperm cell concentration, semen was diluted five times (v/v) with normal saline previously added to it a few drops of formalin (40%) to immobilize the spermatozoa, and used for counting the spermatozoa by an improved Neubauer hemocytometer counting chamber.

### Testicular Histopathology

Specimens from the testes were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70–100%), cleared in xylene, and embedded in paraffin. Five-micrometer thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes [26] and then examined microscopically.

### RNA Isolation, Reverse Transcription

Total RNA was isolated from testis samples using the E.Z.N.A.™ spin column RNA extraction kit (Omega BioTech, category no. R6834-01, Canada) following the manufacturer's instructions. RNA quality was assessed as the 260/280 nm absorbance ratio using the NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then, 0.5 $\mu$g of total RNA was used for production of cDNA using the Qiagen Long Range 2-Step RT-PCR kit (cat. no. 205920).

### Real-Time qPCR

For relative mRNA quantification, real-time quantitative PCR (qPCR) was done according to Takara Bio Inc. guidelines. A total 20 $\mu$L PCR reaction was made by adding 100 ng of cDNA, 1 $\mu$M forward primer, 1 $\mu$M reverse primer, 10 $\mu$L of SYBR Premix Ex Taq with ROX reference (Takara Bio Shiga, Japan), and 6 $\mu$L of nuclease-free water (Ambion, Austin, TX). The reaction was carried out using Rrotor-Gene Q2 plex (Qiagen, Valencia, CA). The thermal profile for real-time PCR was 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each transcript was relatively quantified in three replicates by calculation using the $2^{-\Delta\Delta Ct}$ method [27] for comparison of relative mRNA quantification in testis tissues after normalizing to the housekeeping gene $\beta$-actin. Each sample was repeated three times. Primer sequences and annealing temperatures of the amplified fragments are listed in Table 1

### Statistical Analysis

The obtained data were analyzed using the statistical package for social science (SPSS, version 21) for obtaining mean and standard error. The data were analyzed using one-way analysis of variance to determine the significance of differences among groups. The values were significant at $P \leq 0.05$. Duncan's test was used for making a multiple comparisons among the groups for testing the intergrouping homogeneity.

## RESULTS

### Semen Profile

A semen profile was partially perturbed in rats treated with 100 mg ZnONP (group 3); a significant

**TABLE 2.** Effect of ZnONPs and Querectin on Semen Profile in Male Albino Rats

| *Parameter* | *Group 1* | *Group 2* | *Group 3* | *Group 4* | *Group 5* | *Group 6* |
|---|---|---|---|---|---|---|
| Motility % | $92.5 \pm 4.3^{a}$ | $90 \pm 2.7^{a}$ | $73.3 \pm 5.1^{b}$ | $10 \pm 7.2^{d}$ | $80 \pm 4.1^{ab}$ | $60 \pm 3.3^{c}$ |
| Sp. C.C. | $31.25 \pm 5.1^{a}$ | $25 \pm 4.8^{a}$ | $32.5 \pm 6.2^{a}$ | $1.5 \pm 4.0^{c}$ | $28.2 \pm 6.1^{a}$ | $16.25 \pm 4.3^{b}$ |
| Alive % | $96 \pm 2.3^{a}$ | $87 \pm 4.4^{ab}$ | $80 \pm 2.7^{b}$ | $12 \pm 5.3^{d}$ | $84 \pm 3.9^{ab}$ | $64 \pm 1.3^{c}$ |
| Normal % | $91 \pm 2.5^{a}$ | $88 \pm 4.8^{a}$ | $81 \pm 4.3^{ab}$ | $5 \pm 3.4^{d}$ | $85 \pm 4.7^{ab}$ | $70 \pm 2.1^{c}$ |

Group 1, control group; group 2, querectin group; group 3, zinc oxide nano 100 mg; group 4, zinc oxide nano 400 mg; group 5, zinc oxide nano 100 + querectin; and group 6, zinc oxide.
Sperm Cell Concentration (Sp.C.C.) Sp. C. C. = N $\times$ $10^6$ /mL semen.
Means within the same row and bearing different superscripts are significantly different at $P < 0.05$.
nano 400 + querectin.

decrease in sperm motility and live percentage was observed in the treated group when compared with the control group (Table 2). In the group treated with ZnONP together with querectin (group 5) showed no difference when compared with the control group. In addition, a significant reduction in the mean of sperm cell concentration, motile sperms, and viable sperms with a significant increase in abnormal sperms in the group treated with 400 mg ZnONP (group 4) was observed (Table 2); however, this perturbation was significantly alleviated when the rats were supplemented with ZnONP together with querectin (group 6).

## Lipid Peroxidation, Oxidative Stress, and Antioxidants Activity

Similarly, the level of MDA, as a lipid peroxidation and oxidative stress marker, showed a significant increase in group 4 than other groups whereas a significant decline is observed in group 6 where rats were supplemented with querectin (Table 3). On the other hand, the level of GSH was significantly increased in the querectin-supplemented group. In addition, the activity of glutathione peroxidase (GPx), SOD, and CAT showed a significant increase in group 6 when compared with ZnONP-treated groups (Table 3). Moreover, mRNA expression of CAT and SOD showed a similar behavior to their activities, i.e. significantly increased after supplementation with querectin (Figures 1A and 1B).

## Testosterone Level and Steroidogenesis Pathway

The serum testosterone level showed a significant decrease after treatment with ZnONP; however, it showed a significant increase after supplementation with querectin (Table 4). Similarly, mRNA of 3$\beta$-HSD and 17$\beta$-HSD showed a significant increase in their expression in group 6 when compared with ZnONP-treated groups (Figures 2A and 2B). In addition, Nr5A1 mRNA expression in groups 5 and 6 showed a significant increase when compared with ZnONP-treated groups (Figures 2C).

## Testicular Histopathology

In the control group; normal testicular tissues were observed in the testes of control rats with uniform seminiferous tubules and complete spermatogenesis. The epithelium of testicular tubules was intact containing Sertoli cells resting on the basement membrane, together with spermatocytes and spermatogonia. Spermatids with different shapes were observed embedded in or associated with the Sertoli cells at different stages of the spermatogenic cycle (Figure 3A).

In the querectin-treated group, normal testicular tissues with improvement of spermatogenesis that was represented by the presence of elongated spermatids and huge numbers of spermatozoa in all the lumina of the seminiferous tubules were observed (Figures 3B and 3C).

In rats treated with ZnONP, degenerative changes, atrophy, and necrosis were observed in the majority of the seminiferous tubules. These changes were increased gradually with the increasing the dose. The testes in group 3 that received 100 mg/kg body weight of ZnONPs showed focal mild testicular degeneration of single or several layers of vacuolated spermatocytes besides congested interstitial blood vessels (Figure 3D). Few desquamated spermatocytes were seen in the lumen of some seminiferous tubules (Figure 3E).

The testes in group 4 that received 400 mg/kg B.WT of ZnONPs showed more prominent lesions that were represented by shrunken, disorganized seminiferous tubules with irregular basement membrane and incomplete spermatogenesis. Some tubules showed coagulative necrosis (Figures 3F and 3G), Moreover, the seminiferous tubules were almost empty from spermatids and spermatozoa.

While rats received ZnONPs together with querectin showed normal testicular tissue with intact

**TABLE 3.** Means ± Standard Error of MDA, GSH, GPx, SOD, and CAT in Testicular Tissue of Adult Male Rats

| *T* | *Group 1* | *Group 2* | *Group 3* | *Group 4* | *Group 5* | *Group 6* |
|---|---|---|---|---|---|---|
| MDA (nmol/g tissue) | $22.32 \pm 2.16^{a}$ | $21.26 \pm 2.26^{e}$ | $35.12 \pm 2.32^{b}$ | $44.65 \pm 2.46^{a}$ | $23.61 \pm 2.12^{d}$ | $27.97 \pm 2.43^{c}$ |
| GSH (mg/g tissue) | $14.26 \pm 1.15^{a}$ | $15.13 \pm 1.62^{a}$ | $10.73 \pm 1.12^{d}$ | $7.21 \pm 1.29^{e}$ | $12.7 \pm 1.46^{b}$ | $11.97 \pm 1.76^{b}$ |
| GPx ($\mu$mol NADPH/g tissue) | $19.7 \pm 1.12^{a}$ | $19.06 \pm 1.14^{a}$ | $17.1 \pm 1.06^{c}$ | $14.21 \pm 1.24^{d}$ | $18.12 \pm 0.95^{b}$ | $16.9 \pm 1.09^{c}$ |
| SOD (U/g tissue) | $42.29 \pm 2.6^{a}$ | $43.31 \pm 2.12^{a}$ | $21.73 \pm 3.3^{d}$ | $14.96 \pm 2.15^{e}$ | $34.17 \pm 2.7^{b}$ | $30.97 \pm 2.25^{c}$ |
| CAT ($\mu$mol $H_2O_2$ decomposed/g tissue | $48.9 \pm 3.51^{a}$ | $47.3 \pm 2.65^{a}$ | $38.7 \pm 1.58^{d}$ | $30.7 \pm 1.1^{e}$ | $44.3 \pm 2.65^{b}$ | $41.3 \pm 1.84^{c}$ |

Group 1, control group; group 2, querectin group; group 3, Zinc oxide nano 100 mg; group 4, zinc oxide nano 400 mg; group 5, zinc oxide nano 100 + querectin; and group 6, zinc oxide nano 400 + querectin.
Means within the same row and bearing different superscripts are significantly different at $P < 0.05$.

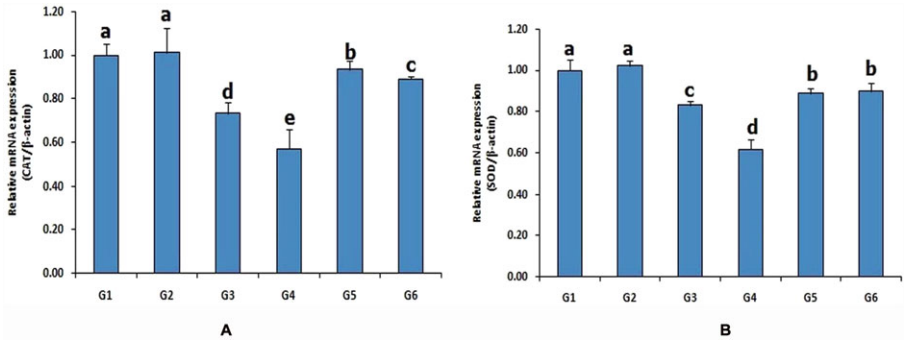


**FIGURE 1.** Relative quantification of CAT and SOD mRNA transcripts in testis of different experimental groups. The expression of the target gene was normalized to the $\beta$-actin expression. The experimental groups were compared with the control group.

**TABLE 4.** Serum Testosterone Levels (ng/mL) in Rats Treated with ZnONP and/or Querectin

| *T* | *Group 1* | *Group 2* | *Group 3* | *Group 4* | *Group 5* | *Group 6* |
|---|---|---|---|---|---|---|
| Testosterone (ng/mL) | $3.26 \pm 0.035^{a}$ | $3.32 \pm 0.061^{a}$ | $2.97 \pm 0.021^{c}$ | $2.12 \pm 0.029^{e}$ | $3.17 \pm 0.026^{b}$ | $2.83 \pm 0.036^{d}$ |

Group 1, control group; group 2, querectin group; group 3, Zinc oxide nano 100 mg; group 4, zinc oxide nano 400 mg; group 5, zinc oxide nano 100 + querectin; and group 6, zinc oxide nano 400 + querectin.
Means within the same row and bearing different superscripts are significantly different at $P < 0.05$.

seminiferous tubules and regular basement membrane and normal spermatocytes and spermatids (Figures 3H and 3I). By increasing the ZnONPs doses, querectin ameliorated the deleterious effect of ZnONPs toxicity as indicated by mild testicular degeneration with few desquamated spermatocytes in the lumen of seminiferous tubules (Figure 3J).

## DISCUSSION

ZnONPs are used in different applications including cosmetics, paints, as drug carrier and filling in medical materials [28]. Direct and indirect release of these nanoparticles into the environment through bathing, sewage effluent, and other engineering application leads to damage of some vital organs of mice such as heart, lung, liver, and kidney [7, 29]. In the current study, we evaluated the toxicological effect of ZnONPs administration to experimental rats on different biochemical and histopathological parameters of male fertility and studied the effect of querectin as a potential antioxidant to ameliorate this toxic effect.

In this study, epididymal sperm parameters including sperm number, motility, and percentage of live/dead and abnormality were also significantly changed in ZnONP-125– and ZnONP-300–treated groups. Gromadzka-Ostrowska et al. showed that even small amounts of silver nanoparticles (NPs) have a toxic impact on the germ cells and reduced sperm quality [30]. Moreover, Talebi et al. showed a significant decrease in epididymal sperm number, motility, and percentage of normal and live sperms in 50 and 300 mg/kg ZnONPs-supplemented mice [4]. Several studies showed the ability of different kinds of NPs to pass through the blood–testis barrier and exert their toxic action on spermatogenesis [31–35].

The testicular architecture showed marked testicular histopathological changes represented by increased cell apoptosis and seminiferous tubular damage and massive sloughing of immature germ cells from the

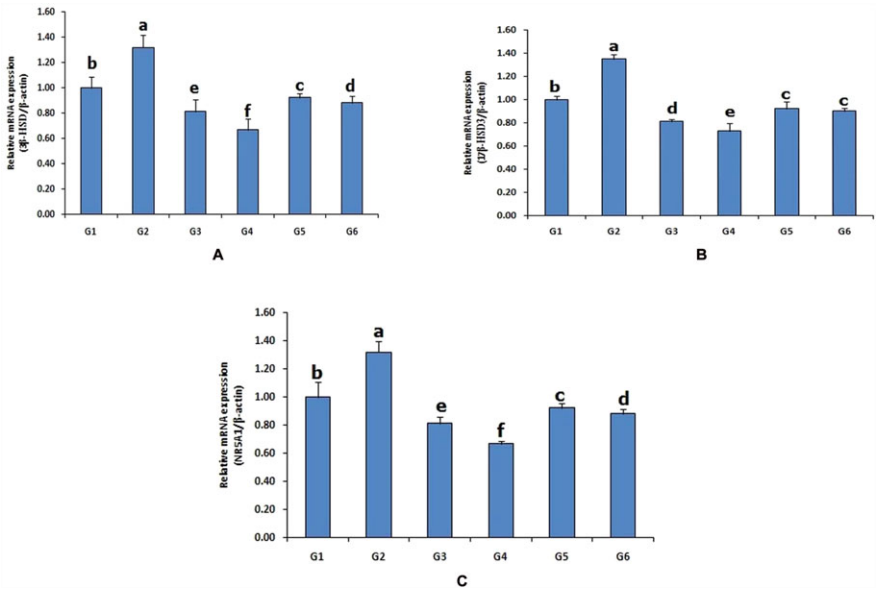


**FIGURE 2.** Relative quantification of 3$\beta$-HSD, 17$\beta$-HSD, and NR5A1 mRNA transcripts in testis of different experimental groups. The expression of the target gene was normalized to the $\beta$-actin expression. The experimental groups were compared with the control group.

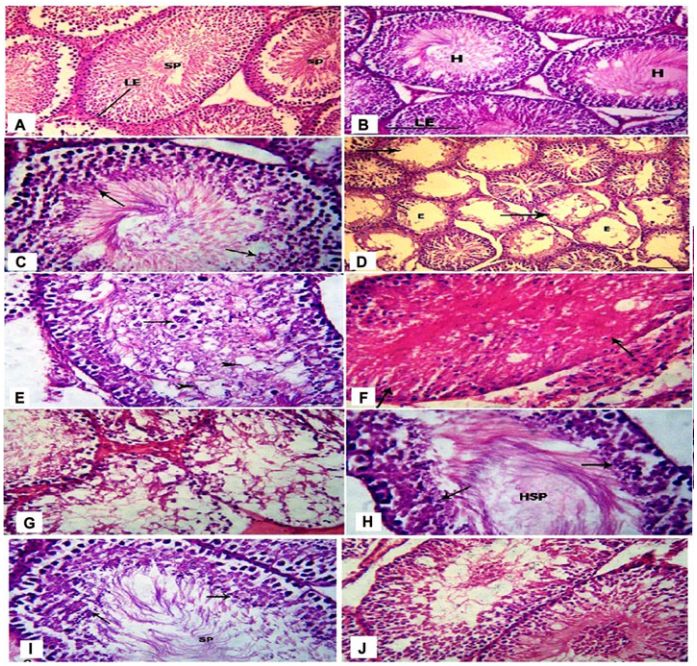


**FIGURE 3.** Photomicrographs of testicular histopathological section of experimental groups: (A) control group; (B) and (C), querectin-treated group; (D) and (E), 100 mg ZnONP-treated group; (F) and (G). 400 mg ZnONP-treated group; (H) and (J), querectin supplementation with ZnONP treatment groups. 40×. H&E staining.

seminiferous tubules that would explain the significant decrease in live and normal sperms in rats treated with ZnONPs.

Moreover, the testosterone level showed a significant decrease in ZnONP-treated groups, a result which is consistent with the decrease in Leydig cell proliferation and activity that was recorded in the present study and may be caused by the direct effect of ZnONPs on Leydig cells. In addition, 3$\beta$-HSD and 17$\beta$-HSD mRNA expression showed a significant decrease in ZnONP-treated groups that explains the direct interference of ZnONP with the steroidogenesis enzymes that are responsible for conversion of cholesterol into testosterone: 3$\beta$-HSD [36, 37] and 17$\beta$-HSD

[38, 39]. Furthermore, Nr5A1 or the steroidogenic factor-1 showed a significant increase after querectin treatment. Nr5A1 regulates several cytochrome P450 steroid hydroxylases in Leydig cells (such as 3$\beta$-HSD and aromatase) and is involved in the steroidogenesis pathway and synthesis of testosterone [40, 41].

Moreover, the expression of CAT and SOD mRNA was significantly decreased and accordingly the levels of antioxidants CAT and SOD as well as GPx were significantly decreased. Lipid peroxidation was significantly increased, as indicated by the MDA level. All these results indicated the ZnONP might be considered as an oxidative stress factor. Interestingly, the rats that were supplemented querectin together with ZnONPs showed less toxic effects or even no toxic effect in most of studied parameters.

Recent studies suggested different mechanisms of ZnONP in causing oxidative stress either in vivo through accumulation of nanoparticles in the affected tissue [7, 42, 43] or in vitro through induction of a range of cytotoxic responses [44] or by release of $Zn^{++}$ ions and increasing reactive oxygen species production [45]

The exact mechanism of ZnONP effects on the testis is not yet known; however, our results would suggest a possible oxidative stress mechanism exerted by ZnONP and can be alleviated through querectin antioxidant supplementation.

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