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Impact of NiO2 Nanoparticles and Curcumin on Testis Torsion/Detorsion Injury: Role of miR-34 and circRNA 0001518

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Abstract

Background: Testicular torsion is one cause of infertility without proper treatment. In this study, we investigate the effects of NiO2 nanoparticles (NPs) and curcumin on sperm parameters in rats and the expressions of genes involved in the apoptotic pathway, as well as expressions of miR-34 and circRNA 0001518. **Materials and Methods:** Forty-eight rats were randomly divided into eight groups: control (healthy rats), control rats that received NiO2-NPs, healthy rats that received curcumin, rats that received simultaneous NiO2-NPs and curcumin, untreated testicular ischemia/reperfusion (I/R) rats, testicular I/R rats that received NiO2-NPs, testicular I/R rats that received curcumin, and testicular I/R rats that received NiO2-NPs and curcumin. Then, sperms were extracted from the rats' epididymides to analyze concentration, viability, morphology, and motility. The cellular apoptosis level was studied using flow cytometry. Also, Bad and Bcl-X gene expressions and miR-34 and circRNA 0001518 levels were measured. **Results:** We observed improved sperm parameters in the testicular I/R) rats that received curcumin and NiO2-NPs. Administration of NiO2-NPs to healthy rats increased both apoptosis and the Bad/Bcl-X expression ratio. However, its administration to testicular I/R rats alone or with curcumin decreased apoptosis and the Bad/Bcl-X expression ratio and increased expressions of miR-34 and circRNA 0001518. **Conclusion:** Administration of NiO2-NPs and curcumin, alone or in combination, can have therapeutic effects in testicular I/R conditions by altering the expressions of genes in the mitochondrial apoptotic pathway and their regulatory elements. **[GMJ.2021;10:e2342] DOI:10.31661/gmj.v10i0.2342**

Keywords: Testicle; Torsion; Rat; *Bcl-x*; miR-34; *Bad*; circRNA

Introduction

The most common cause of infertility in men is their inability to produce healthy sperm. Testicular torsion is an emergency condition that occurs due to testis torsion around the vertical axis of the spermatic cord [1], which disturbs testicular blood

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circulation and eventually leads to edema and disturbances testicular circulation [2]. Ischemia/reperfusion (I/R) damage increases free radical production, interferes with spermatogenesis, and reduces sperm counts [3].

Curcumin, the main active compound of the turmeric plant [4], is a yellow phenolic compound that has a wide range of biological and pharmacological activities [5, 6]. This substance's most important biological effects are its anti-inflammatory and anti-tumor properties [7]. In addition, curcumin is a popular antioxidant and one of the most potent free radical scavengers that can prevent the production of reactive oxygen species (ROS) [5]. The results of many studies show the antioxidant properties of curcumin and its protection of the male reproductive system against a variety of environmental hazards and inducers of oxidative stress [8, 9].

Due to the high antioxidant properties of curcumin, this compound can be used against oxidative damage caused by testicular I/R injury [10].

The production and consumption of nanoparticles (NPs) have increased concerns about their harmful side effects on human health. Although some researchers consider NPs non-toxic compounds, others have reported their toxic effects [1, 12]. Nickel NPs are spherical black particles with a large surface area [13]. The proposed mechanism of action of nickel is via the formation of irreversible bonds with macromolecules, which disrupt the biological activities of cells [14]. Although recent reports indicate that many NPs have detrimental or toxic effects on spermatogenesis, some NPs have non-toxic or beneficial impacts on this process. These reports suggest that the animal species, drug use, NP dose, and its characteristics play an essential role in determining the effects of NPs on spermatogenesis. The ability of NPs to penetrate the testicular blood barrier plays a vital role in explaining the toxicity of NPs on spermatogenesis [15].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. As soon as mRNA binds to the target mRNA, the inhibition of protein expression will occur [16]. One of the miRNAs involved in the apoptotic process is miR-34, which has a pro-apoptotic role (17). The expression of this miRNA is regulated by Bcl-2 (anti-apoptotic protein) expression; inhibition of Bcl-2 plays an essential role in inducing miR-34 expression [17]. This miRNA increases the expressions of the CREB, BIRC5 (Survivin), and YY1 apoptotic proteins, which increases apoptosis in cells [18, 19]. miR-34 expression is essential for the activity of the pro-apoptotic protein P53 [20].

Circular RNAs (circRNAs) appear to act as sponges for miRNAs, leading to gene expression changes [21]. circRNAs can form covalent bonds and create continuous loops. Researchers are interested in identifying the circRNAs involved in various biological processes, which can be greatly important [22, 23].

Thus, in the current research, we investigated the impacts of curcumin and NiO2 NPs (NiO2-NPs) on reducing testicular I/R injury. The expressions of genes involved in mitochondrial apoptosis and miR-34 and circRNA 0001518 were also evaluated.

Materials and Methods

Materials

NiO2-NPs and curcumin were obtained from Merck (Germany). The NiO2 concentration was calculated based on the mean lethal concentration (0.02 mg/kg). Injectable curcumin was provided by the Antiaging Institute (CA, USA).

The Wistar rats were allowed to acclimate to their surroundings before any experimental procedures. All of the 48 rats (weight: 250-300 g) from Pasteur Institute (Tehran, Iran) were exposed to a light/darkness cycle of 12 h, a temperature of 25°C, and 55% relative humidity (RH). The rats had free access to food and water. Their diet consisted of equal proportions of wheat, corn, and barley. The animals were also allowed to adapt to the laboratory conditions for one week. All tests were performed from 09:00 to 15:00 to prevent the circadian effect.

Induction of Testis Torsion/Detorsion

The rats were anesthetized with ketamine(10mg/ml) and xylazine (20 mg/ml). Next, induction of testicular torsion/detorsion was performed by twisting the testicles 720° counterclockwise for 90 min. Oligoasthenoteratozoospermia was confirmed by testicular pathology analysis. This operation was performed for 50 days, and during this time, the rats were treated with NiO2-NPs and curcumin.

Animal Groups

We randomly divided the animals into the following eight groups: (1) healthy rats (control; n=6); (2) healthy rats that received NiO2-NPs (n=6); (3) healthy rats that received curcumin (n=6); 4) rats that received simultaneous NiO2-NPs and curcumin (n=6); (5) untreated testicular I/R rats (n=6); (6) testicular I/R rats that received NiO2-NPs (n=6); (7) testicular I/R rats that received curcumin (n=6); and (8) testicular I/R rats that received NiO2-NPs and curcumin (n=6). All the used procedures were strictly followed according to the guidelines of the ethics committee approved by Islamic Azad University, North Tehran Branch under the code of ethics committee: 15748005726641700.

Sperm Analysis

First, all sperm collection equipment was heated to 37°C with a hot plate. Then, the animals were killed with an injection of sodium thiopental, and their testes were isolated. The epididymis tissues were separated under sterile conditions, cut into small pieces, placed in 5 mL HBSS medium with 5 mg/ml bovine serum albumin, and incubated at 37°C for 20 min.

Sperm viability was measured using an eosin-nigrosin stain. For this purpose, 20 µl of sperm was added to 10 µl of 0.05% eosin-nigrosin stain. The dead sperm stain pink due to the destruction of their plasma membranes, whereas live sperm do not absorb the stain. The slides were examined under an optical microscope at 400x magnification.

Sperm motility was determined by placing 10 µl of the sperm solution onto a microscope slide and examining it with a light microscope at 400x magnification [24].

Sperm morphology was evaluated with standard Papanicolaou staining [25], and the slides were observed under an optical microscope (400x). For this purpose, we examined 200 sperms for the presence of any abnormal morphology that included sperm with two heads, large head, small head, round head, no acrosome, no head, long or short tail, no tailor twisted tail. The cytoplasmic diameter was also assessed.

Viability Assay

For survival measurement, the MTT assay was used. In this regard, 5 mg of MTT (2,5-diphenyltetrazolium bromide, Sigma Aldrich) was dissolved in 1 ml of phosphate buffer. The solution was made at a 5 mg/ml concentration and stored in the refrigerator for use. 10 to 20 mg of testicular tissue was lysed in PBS buffer by a homogenizer and then centrifuged at 4°C (12 minutes at 12,000 rpm). Then 50 µl of MTT solution was added to the tubes and incubated for 2 hours at 37 ° C. After 2 hours, 500 µm DMSO was added to each tube and shaken well to dissolve all Informazione crystals. The solution was then transferred to a 96-well, and 1 hour later, the absorption was read at 560 nm, and the reference wavelength was 630 nm with calibration of 1.99 by a multi-plate device.

Apoptosis Measurement

Testicular cells were obtained by enzymatic digestion of the epididymides in collagenase for 4 h at 37°C. Fetal serum was added to inactivate the enzyme, then centrifuged the cells; the cell suspension was filtered and transferred into plates. Then, 10 ml of DMEM medium, contained 3% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 3% non-essential amino acids were added to the plates and incubated at 37°C and 5% CO2., Flow cytometry was used to evaluate necrosis and apoptosis in the cells. Annexin V-FITC staining was used to show apoptosis, and simultaneous staining with propidium iodide (PI) as a marker was used to evaluate necrosis and apoptosis. In this regard, after PBS wash

Table 1. Primer Sequences Used in The Current Study to Amplify the Bad, Bcl-x, miR-34 and CircRNA 0001518 Genes.

Genes	Sequence (3'-5')
rat-*Bcl-X-F*	GCTGGTGGTTGACTTTCTCTCC
rat-*Bcl-X-R*	GGCTTCAGTCCTGTTCTCTTCG
rat-*Bad-F*	GGAGCATCGTTCAGCAGCAG
rat-*Bad-R*	CCATCCCTTCATCTTCCTCAGTC
miR-34-F	Ordered from Pars Zengan Company Code PG-1
circRNA 0001518	Ordered from Pars Zengan Company Code PG-1
rat-*β-Actin-F*	CGGTTCCGATGCCCTGAGGCTCTT
rat-*β-Actin-R*	CGTCACACTTCATGATGGAATTGA

and centrifugation, 100 µl of binding buffer was added to the testis cells treated with NiO2-NPs and curcumin at LD50 concentration, then incubated with 5 µl Annexin V-FITC (Sigma Aldrich) in the dark at 4°C for 15 minutes. Followed by washing and re-centrifugation, 10 µl of PI (10 ml / 100 ml PBS) (Sigma Aldrich) was added to the cell precipitate, then flow cytometry was performed using a Partec GmbH flow cytometer (Partec PA S, Germany).

RNA Extraction and cDNA Synthesis

We used an RNA Extraction Kit (Yekta Tajhiz Azma, Tehran, Iran) to extract RNA from the testicular tissue. Gel electrophoresis and a nanodrop device were used to evaluate the quality and quantity of the extracted RNA, respectively. cDNA synthesis was performed using a cDNA Synthesis Kit (Yekta Tajhiz Azma, Tehran, Iran) based on the manufacturer's instructions. Quantitative measurement of cDNA was performed with a nanodrop device.

Primers

The primers for the *Bad*, *Bcl-X* genes were designed using Gene Runner software. miR-34 and circRNA 0001518 primers have been purchased from the company (Ordered from Pars Zengan Company Code PG-1). Table-1 lists the primer sequences used in the current research. The RT-PCR (ABI 7300) timing and temperature program started at 95 °C for 30 s for cDNA denaturation, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 31 min. In the next step, the temperature cycles of 95°C for the 15s, 60°C for the 30s, and 95°C for the 15s were used.

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey's multiple range test were used to evaluate significant differences among the groups. Prism 8 software (version 8.0, USA) was used for data analysis. A probability level of $P<0.05$ was considered to be substantial.

Table 2. Correlations Among the Studied Genes.

	Bad	***Bcl-X***	***Bad/Bcl-X***	***circRNA 0001518***	***miR-34***
Bad	1	-0.93113	0.98202	-0.9126	0.97844
Bcl-X	-0.93113	1	-0.98323	0.90951	-0.94779
Bad/Bcl-X	0.98202	-0.98323	1	-0.92713	0.97986
circRNA 0001518	-0.9126	0.90951	-0.92713	1	-0.91337
miR-34	0.97844	-0.94779	0.97986	-0.91337	1

Results

Sperm Parameters

We observed substantial decreases in motility, concentration, sperm with normal morphology, and viability after induction of testis I/R in the rats (Figure-1). Treatment of healthy rats with NiO2-NPs significantly reduced motility, normal sperm counts, sperm concentration, and viability compared to the healthy controls. However, in testicular I/R rats, the combination of NiO2-NPs and curcumin improved all sperm parameters compared to the control testicle I/R rats. In the healthy rats, curcumin significantly improved the numbers of sperm with normal morphology; however, in the testis I/R rats, improvements in all sperm parameters were observed after curcumin administration. (Figure-1).

Apoptosis and Necrosis in the Testis Cells

Induction of testicular I/R damage resulted in a sharp increase in testicular cell necrosis compared with healthy controls. When NiO2-NPs were administered to healthy rats, we observed a three-fold increase in the percentage of testicular cell necrosis compared to healthy control testes. However, administration of NiO2-NPs to rats with testicular I/R damage reduced testicular cell necrosis compared with the torsion/detorsion rats. The strong effect of curcumin on the reduction of necrosis was observed in both the healthy and I/R testis groups, which indicated the protective effects of curcumin against testicular I/R damage. Co-administration of curcumin and NiO2-NPs reduced the percentage of cell necrosis (Figure-2).

Flow cytometry analysis showed increased apoptosis in the healthy rats and a reduction in apoptosis in the testis I/R rats after treatment with the NiO2-NPs. Also, simultaneous treatment of NiO2-NPs with curcumin reduced apoptosis (Figure-3).

Gene Expression Analysis

NiO2-NPs in healthy rats and testicular torsion/detorsion resulted in overexpression of the *Bad* gene and downregulation of *Bcl-X* gene expression. However, curcumin downregulated *Bad* gene expression and overexpressed *Bcl-X* in healthy and testicular I/R rats. Co-administration of NiO2-NPs with curcumin decreased *Bad* gene expression and increased *Bcl-X* expression. These results indicated the pro-apoptotic effects of NiO2-NPs and the anti-apoptotic effects of curcumin (Figure-4).

In healthy rats that received NiO2-NPs, we observed upregulation of miR-34 and downregulation of circRNA 0001518 compared with healthy control rats. However, the administration of curcumin resulted in the downregulation of miR-34 and upregulation of circRNA 0001518. On the other hand, testicular I/R induction resulted in significant overexpression of miR-34 and downregulation of circRNA 0001518 expressions. Administration of NiO2-NPs to the testis I/R rats caused overexpression of miR-34 and downregulation of circRNA 0001518 (Figure-5a, b).

The present study results showed a significant negative correlation between *Bad* gene expression and *Bcl-X* (r=-0.931) and circRNA 0001518 (r=-0.912). However, there was a significant positive correlation between *Bad* gene expression and miR-34 (r=0.978). A significant positive correlation also existed between *Bcl-X* gene expression and circRNA 0001518 (r=0.909); however, there was a significant negative correlation between *Bcl-X* gene expression and miR-34 (r=-0.949). A significant negative correlation (r=-0.913) was observed between miR-34 expression and circRNA 0001518 (Table-2).

Discussion

Testicular I/R injury is caused by a loss of blood supply to the testicles, which rapidly causes cell death and damage [26]. Many factors are involved in I/R cell death, such as increased ROS, free radicals, lipid peroxidation, increased inflammatory cytokines, and damage to blood vessels [27]. Although there is no definite preventative treatment, recently proposed interventions reduce this damage include blocking the production of free radicals, anti-inflammatory drugs, angiotensin-converting enzyme inhibitors,

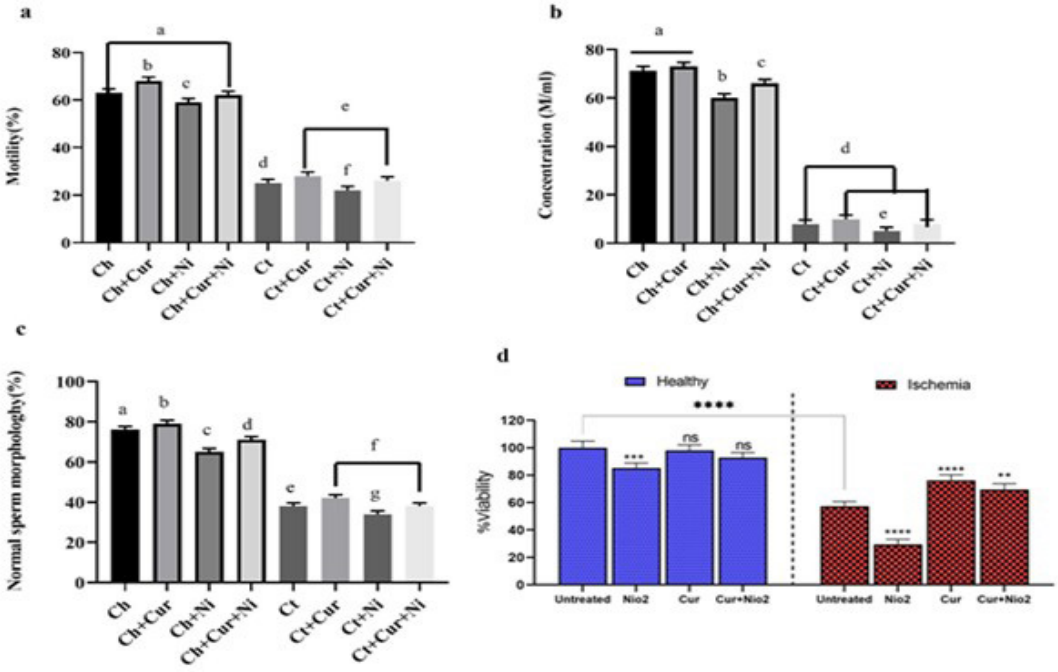

Figure 1. The impact of curcumin and NiO2 nanoparticles (NiO2-NPs) on sperm motility (a), concentration (b), morphology (c), and viability (d) in the different rat groups.
*Different letters indicate significant differences between the groups according to the Tukey test at a probability level of P<0.05. **, ***, and ****: Indicate significant differences among groups at probability levels of P<0.01, P<0.001, and P<0.0001, respectively. **Ch:** Healthy control; **Ch+Cur:** Healthy control that received curcumin; **Ch+NiO2:** Healthy control that received NiO2-NPs; **Ch+Cur+NiO2:** Healthy control that received curcumin and NiO2-NPs; **Ct:** Rats with testicular ischemia/reperfusion (I/R); **Ct+Cur:** Rats with ischemic testicles that received curcumin; **Ct+NiO2:** Rats with ischemic testicles that received NiO2-NPs; **Ct+Cur+NiO2:** Rats with ischemic testicles that received curcumin and NiO2-NPs.

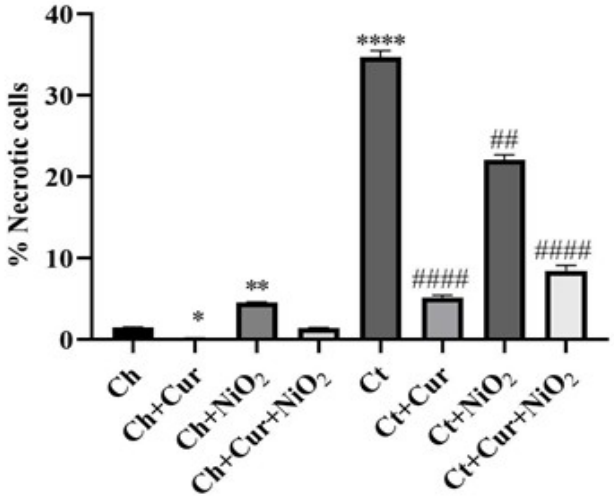

Figure 2. Percentage of necrosis in testicular cells of healthy and testicle torsion/detorsion rats treated with NiO2 nanoparticles (NiO2-NPs) and curcumin. *, **, and ****: Indicate significant differences compared with the healthy control at probability levels of P<0.05, P<0.01, and P<0.0001, respectively. ## and ####: Indicate significant differences compared with ischemia/reperfusion (I/R) rats at probability levels of P<0.01 and P<0.0001, respectively. **Ch:** Healthy control; **Ch+Cur:** Healthy control that received curcumin; **Ch+NiO2:** Healthy control that received NiO2-NPs; **Ch+Cur+NiO2:** Healthy control that received curcumin and NiO2-NPs; **Ct:** Rats with testicular ischemia/reperfusion (I/R); **Ct+Cur:** Rats with ischemic testicles that received curcumin; **Ct+NiO2:** Rats with ischemic testicles that received NiO2-NPs; **Ct+Cur+NiO2:** Rats with ischemic testicles that received curcumin and NiO2-NPs.

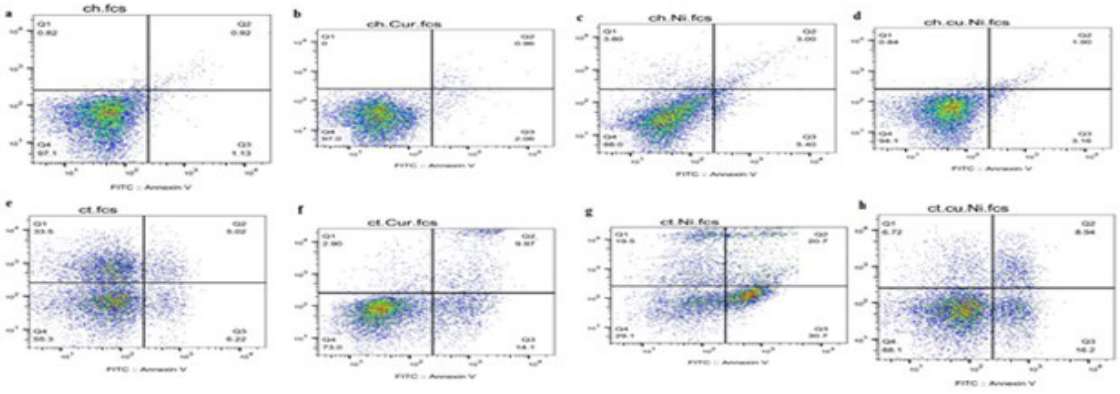

Figure 3. Flow cytometry assessment of apoptosis in testis cells from healthy rats (a), healthy rats that received curcumin (b), healthy rats that received NiO2 nanoparticles (NiO2-NPs) (c), healthy rats that received curcumin and NiO2-NPs (d), testicular ischemia/reperfusion (I/R) rats (e), testicular I/R rats that received curcumin (f), testicular I/R rats that received NiO2-NPs (g), and testicular I/R rats that received curcumin and NiO2-NPs (h).

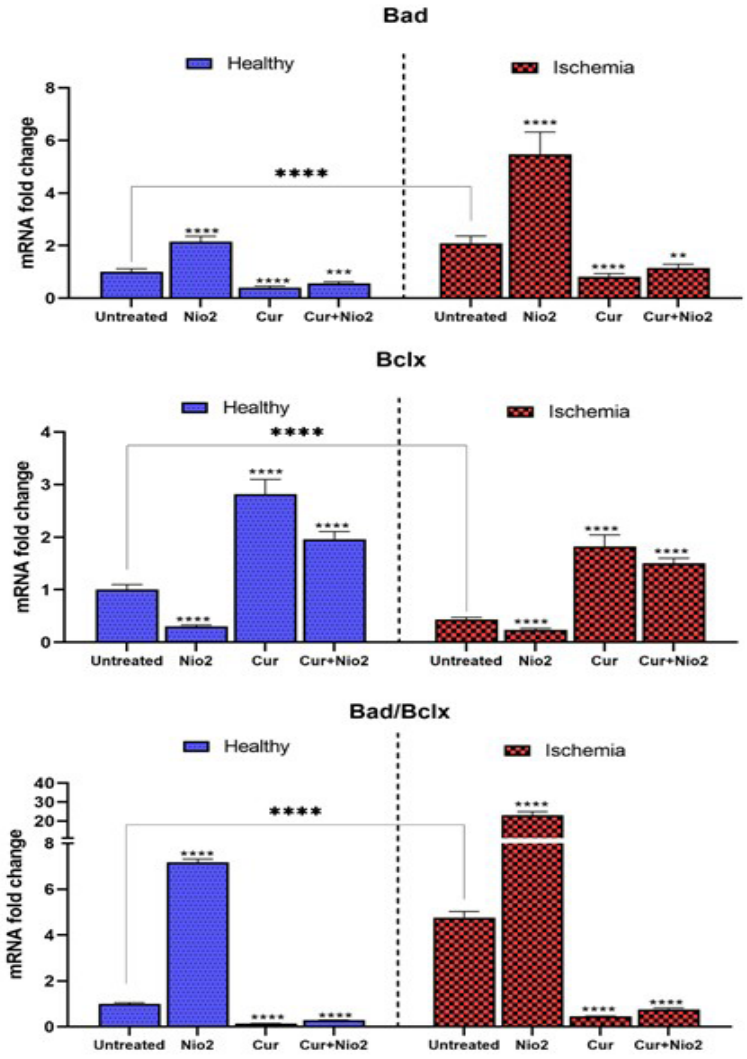

Figure 4. Expressions of the Bad (a) and Bcl-X (b) genes, and the Bad/Bcl-X-2 ratio (c) in healthy and testis torsion/detorsion rats treated with curcumin and NiO2 nanoparticles (NiO2-NPs). **, ***, and ****: Indicate significant differences at probability levels of P<0.01, P<0.001, and P<0.0001, respectively.

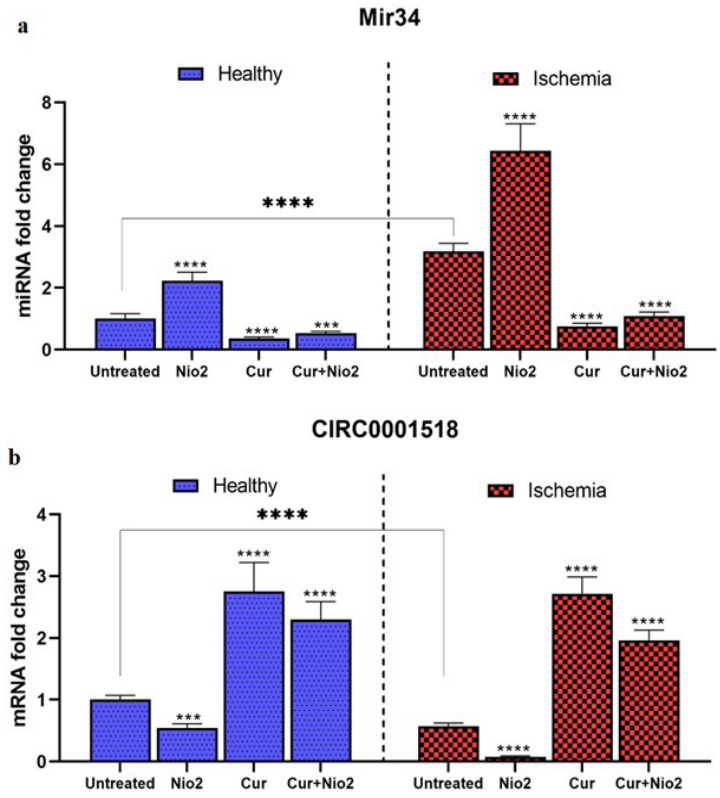

Figure 5. miR-34 and circRNA 0001518 expressions in testicle cells of healthy and ischemia/reperfusion (I/R) rats that received NiO2 nanoparticles (NiO2-NPs) and curcumin. *** and ****: Indicate significant differences at probability levels of P<0.0001 and P<0.0001.

and drugs such as adenosine, morphine, and statins [28]. The results of studies show that antioxidant and free radical scavenging compounds have protective effects on I/R injury [29] . Therefore, in the present study, the sharp decrease in sperm parameters due to testis I/R injury can be attributed to the high production of ROS and apoptosis in these cells.

Antioxidants are an important defense factor against oxidative stress caused by free radicals. Curcumin has antioxidant effects due to its unique structure, including 2-methoxyphenol and enol diketone. The special structure of curcumin can trap free radicals through its antioxidant chain. Due to its antioxidant properties, curcumin can increase sperm motility and viability [30]. Current research results confirm that curcumin can significantly improve sperm parameters in rats with testis I/R. Studies have also shown that curcumin can restore sperm cell structure and function dose-dependent [31]. Therefore, the improvement of sperm parameters due to curcumin in the present study can be attributed to its antioxidant and protective activities on the structure and function of sperm cells [32]. It has been reported that curcumin has protective effects against infertility by improving sperm production, motility, and sperm counts in male pigs exposed to gentamicin [33]. Researchers believe that increasing the level of free radicals and loss of epithelial cells leads to damage to Sertoli cells, destruction of cytoplasmic vesicles, and reduced numbers of sperm with normal morphology [34]. Therefore, in the present study, the increase in the percentage of sperm with normal morphology in testis I/R rats could be attributed to Sertoli cell damage and cytoplasmic vesicle destruction. The antioxidant properties of curcumin could prevent further reductions in the number of sperm with normal morphology.

An important alternative for the chemical

synthesis of NPs is their green synthesis from different plants that have antioxidant properties [35]; therefore, such NPs prevent oxidative damage to cells [36]. The antioxidant properties of various particles, including nano selenium [37], have been reported. The present study showed an improvement in cell damage after administration of NiO2-NPs to testicular I/R rats due to reductions in necrosis and increased cell apoptosis. However, these NPs to healthy rats resulted in increased cell damage. Decreased cellular energy and increased cell death appear to cause cell death in healthy rats. However, in testicular I/R rats, testicular I/R induction resulted in a severe reduction in cellular energy. Treatment with NiO2-NPs slowed this reduction. Therefore, the NPs exhibited antioxidant properties and prevented cell death.

Some of the Bcl-2 family proteins (such as Bcl-2 and *Bcl-XL*) are anti-apoptotic, while others (*Bad*, BAX, or Bid) are pro-apoptotic. Cell susceptibility to apoptotic stimuli may depend on the balance between pro-and anti-apoptotic proteins of the Bcl-2 family [38]. In the present study, overexpression in the *Bad* gene and downregulation of *Bcl-X* expression was observed after testicular I/R injury. It has been shown that *Bad* can bind to *Bcl-XL* more than BAX, and dimerization of *Bad* with *Bcl-XL* results in the displacement of BAX from *Bcl-XL* [39]. Significant reduction in *Bad* levels in testicular I/ R rats that received curcumin showed an inhibitory effect on *Bad* expression. Overexpression of *Bad* was attributed to NiO2-NPs administration in the testes of healthy and I/R rats, which indicated the pro-apoptotic effects of these NiO2-NPs.

Bcl-XL is present in spermatocytes and spermatids in the adult testis [40]. There was a significant increase in *Bcl-XL* at the time of curcumin administration, which indicated the anti-apoptotic effects of curcumin due to its antioxidant properties. The *Bcl-X* gene was first cloned in 1993 based on its similarity to Bcl-2 due to the cDNA capacity of Bcl-2 mice to hybridize to the associated chicken gene [41]. This sequence was then used to isolate the human *Bcl-X* ortholog. In the present study, treatment with curcumin showed a significant increase in *Bcl-X* expression, an important anti-apoptotic factor, in rat testicular tissue. But NiO2NPs showed downregulation of *Bcl-X* expression in both healthy and testis I/R rats, which showed the pro-apoptotic effects of NiO2-NPs.

The present study results showed significant overexpression of miR-34 in I/R testes. Curcumin and NiO2-NPs downregulated the expression of this miRNA. The innovation of the present study is the discovery of miR-34 as a key factor that mediates cell death in damage from testicular I/R. miR-34 plays various roles in many diseases. miR-34 has been reported to suppress carcinogenesis [42] and induce cardiovascular disease, aging, spatial cognitive impairment, and hearing impairment, at least in part through pro-apoptotic action [43]; however, there is less data on the function of miR-34 in testis I/R injury. miR-34 overexpresses under oxidative stress [44]. Oxidative stress is one of the essential mechanisms that cause severe consequences for the testicles and spermatogenesis and can lead to infertility. We observed overexpression of miR-34 in the testes in the presence of increased oxidative stress from the I/R injury. However, curcumin in conjunction with NiO2-NPs could partially prevent its expression.

Recent studies indicate that circRNAs are important biomarkers for disease diagnosis and treatment. The regulatory role of circRNAs in the process of apoptosis has been shown. In myeloma and osteosarcoma cells, circUBAP2 and hsa_circ_0001892 compete for miR-143 and inhibit apoptosis [45, 46]. In the present study, we showed that circRNA 0001518 played an anti-apoptotic role for the first time. Correlation analysis showed that circRNA 0001518 had a significant positive correlation with the *Bad* gene and a significant negative correlation with *Bcl-X*. Therefore, our study suggests that circRNA 0001518 plays a pro-apoptotic role in the I/R testicular disease process.

Conclusion

In the current study, a reduction in testis I/R injury after administration of curcumin along with NiO2-NPs was attributed to reduced

apoptosis, a decreased *Bad/Bcl-X* expression ratio, downregulation of miR-34 and overexpression of circRNA 0001518.

Conflict of Interest

There is no conflict of interest for the current study.

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