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Original article

Protective effects of Curcumin on testicular toxicity induced by titanium dioxide nanoparticles in mice

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ABSTRACT

Objective: In this study, we investigated the preventing effects of Curcumin (Cur) against titanium dioxide nanoparticle ($NTiO_2$)-induced mouse testicular damage.

Methods: We assessed $NTiO_2$-intoxicated mice received 50mg/kg of $NTiO_2$ for 35 days. The Cur + $NTiO_2$ group was pretreated with Cur (200 mg/kg) for 7 days prior to administering $NTiO_2$. Sperm parameters, testosterone concentration, histological criteria, morphometric parameters and Johnsen's scoring.

Results: $NTiO_2$ significantly reduced testicular weight, testosterone concentration, morphometric parameters, Johnsen's scoring and sperm quality ($p<0.01$), as well as a significant increase in histological criteria. Pretreatment with Cur reduced testicular weight, ameliorated morphometric parameters, increased Johnsen's scoring, elevated testosterone levels, and increased histological criteria such as vacuolization, detachment, and sloughing of germ cells into the seminiferous tubules. Cur also improved sperm parameters including sperm count, motility, and percentage of abnormality.

Conclusion: Cur was found to have a potent protective effect against spermatogenesis defects induced by nanoparticles in mice.

Keywords: curcumin, nanoparticle, spermatogenesis, testosterone, sperm quality, mice

INTRODUCTION

Many recent *in vivo* studies have shown that most nanoparticles (NPs) have toxic effects on male germ cells (Iavicoli *et al*., 2013; Brohi *et al*., 2017). NPs have the capacity to cross the blood-testis barrier and induce testicular toxicity (Lan & Yang, 2012). Among the various metal nanomaterials, titanium dioxide NPs ($NTiO_2$) is used in a variety of consumer products such as sunscreens, cosmetics, clothing, electronics, paints and surface coatings (Cox *et al*., 2016). $NTiO_2$ has been shown to cause reproductive toxicity including spermiation failure, low sperm production, and abnormal sperm morphology in mice (Miura *et al*., 2014). *In vitro* studies have also shown that $NTiO_2$ induces cellular toxicity in the reproductive system (Hong *et al*., 2016). $NTiO_2$ can be absorbed by spermatids, Sertoli cells, and Leydig cells, and induce histological changes in seminiferous tubules, causing damage to sperm production, sperm motility, and Sertoli cell number (Gao *et al*., 2013).

Since the high-rate use of NPs, such as $NTiO_2$, affects male reproduction or male reproductive function, it seems crucial to find a suitable drug for reducing its toxic effects. Curcumin (Cur), a yellow pigment present in the rhizome of turmeric (*Curcuma longa*), has several pharmacological properties including anti-inflammatory, anti-carcinogenesis, antioxidant, and hypocholesterolemic activities (Hewlings & Kalman, 2017). Previous studies showed beneficial effects of Cur against testis damages. For example, Cur inhibited the testicular damage induced by alcohol, cisplatin, aflatoxin, metronidazole, ischemia reperfusion and cadmium (Giannessi *et al*., 2008; Ilbey *et al*., 2009; Mathuria & Verma, 2007; Noorafshan *et al*., 2010; Wei *et al*., 2009; Salama & El-Bahr, 2007). Therefore, we performed the present study to evaluate the preventive effects of Cur on $NTiO_2$-induced testicular damage in the mouse.

MATERIAL AND METHODS

Animals

Thirty-two NMRI mice (6-8 weeks, 25-30 g) were used in this study. The mice were kept under a light-dark cycle of 12:12 at a temperature of 22 ± 3°C and a humidity of 50%±5%. *They were* given *free access* to water and pellets (commercial food). Our study was approved by the Ethics committee of the Ahvaz Jundishapur University of Medical Sciences (approved number: IR.AJUMS.REC. 1393s98).

Experimental design

The study mice were randomly assigned to 4 groups (8 animals in each group) described below (Fig. 1):

Group 1 (Control): received normal saline (0.2 ml) for 42 days.

Group 2 (Cur): was treated with Cur (200 mg/kg) for 42 days (Al-Rubaei *et al*., 2014),

Group 3 ($NTiO_2$): was administered first by normal saline (0.2 ml) for 7 days and then simultaneously with $NTiO_2$ (50mg/kg) for 35 days (Song *et al*., 2017).

Groups 4 (Cur + $NTiO_2$): received first 200 mg/kg of Cur for 7 days followed by concomitant administration of 50mg/kg $NTiO_2$ for 35 days.

All treatments were given by gavage. Twenty-four hours after the last administration, the mice were sacrificed, and the testicles from each animal were dissected, weighed and maintained in 10% formalin. Paraffin sections (5 µm) were prepared and stained with hematoxylin and eosin (H&E) for histology, Johnsen's scoring and morphometric evaluations.

Nanoparticle preparation

The $NTiO_2$ (Sigma-Aldrich Co.) was diluted by mili-Q water (Song *et al*., 2017). A dynamic light scattering approach of Zetasizer-Nano-ZSP (Malvern, UK) was used to analyze the mean particle size distribution, polydispersity index (PDI) and zeta potential. The mean particle size was 68.4±5.7. The zeta potential value was measured to be +26.2 mV, high enough to make the NPs repel each other and prevent particle aggregation. The PDI value was calculated to be 0.19, showing an excellent homogeneous ZNP size distribution.

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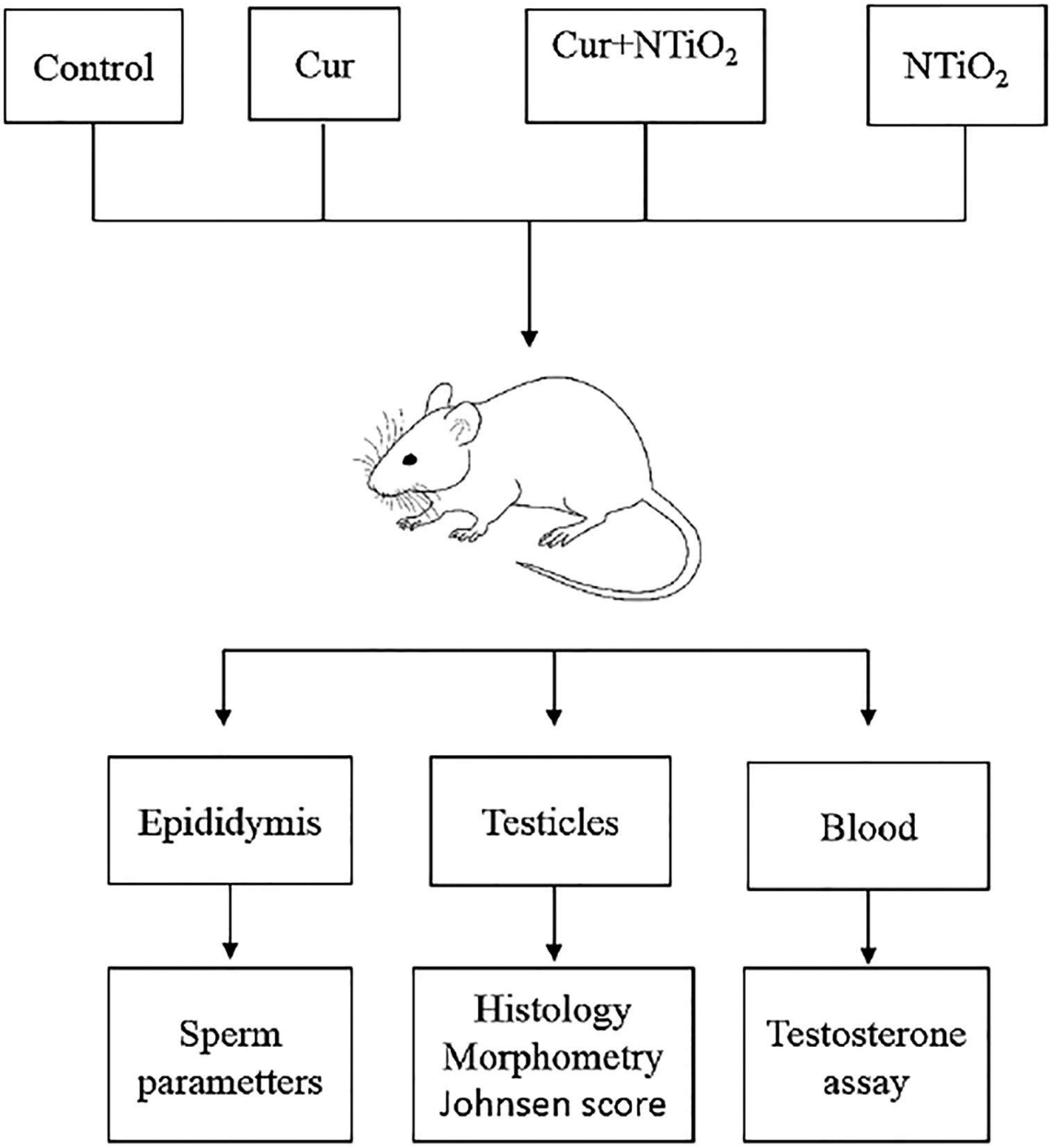

Figure 1. Schematic illustration of experimental design.

Testosterone assay

The blood samples were collected from the mice heart and centrifuged to obtain serum. Serum testosterone concentration was measured using a commercial Testosterone ELISA kit (SE120089, Sigma, St *Louis, MO* , USA) *according* to *manufacturer's* instructions.

Morphometry

The diameters of the seminiferous tubules and the lumen diameter were measured using the Mutic Images Plus 3.0 software. The height of the seminiferous epithelium was calculated by subtracting the lumen diameter from the tubules' diameter. For each animal, 100 tubules were analyzed.

Histology

Six microscopy slides per animal were examined for signs of testicular damage, including detachment of spermatogenic cells from the basal lamina, sloughing of germ cells and vacuolization in the germinal epithelium. We calculated the average percentage of affected tubules for each feature.

Assessment of spermatogenesis

The maturity of the germinal epithelium was graded using the Johnsen's scoring system (Johnsen, 1970), a simple method for the evaluation of spermatogenesis. In each mouse, 150 tubules were considered and a score ranging from 1 to 10 was given for each tubule. The tubules with complete inactivity were scored as 1 and those with maximum activity (at least 5 or more spermatozoa in the lumen) were scored as 10.

Sperm parameters

Spermatozoa were collected from the right epididymal cauda for examination of sperm motility using

the Computer Assisted Semen Analysis (CASA) system (Hamilton Thorne, USA). The sperms were scored as immotile if no movement was detected. The percentages of rapid progressive, slow progressive and no progressive sperms were evaluated in each sample. A suspension of spermatozoa (from the left epididymal cauda) was prepared and counted using a Neubauer hemocytometer. One drop of the suspension was smeared and observed under a light microscope for morphological assessments (Goodson *et al*., 2011; Hajshafiha *et al*., 2013).

Statistical analysis

The data analysis was performed using the *SPSS* (version 21.0, Chicago, IL, *USA*) one-way analysis of variance (ANOVA), followed by post-hoc pairwise comparison applying the Bonferroni or Mann-Whitney U test. The variables were tested in SPSS for normality distribution and homogeneity. Furthermore, a *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Weight change

No significant differences in body weight were seen between the Control and Experimental groups. The weight of the testicles in the Cur group was slightly higher than that of the Control group. A significant reduction in testicular weight was found in the $NTiO_2$ group ($p<0.05$). In the $NTiO_2$ + Cur-treated animals, a significant increase in testicular weight in comparison with the $NTiO_2$- intoxicated mice ($p<0.05$) was observed (Table 1).

Testosterone assay

As shown in Figure 2, no significant changes were found between the Control and Cur groups in testosterone concentration. The testosterone concentration was significantly reduced in the $NTiO_2$-intoxicated animals ($p<0.01$). There was a significant increase in testosterone levels in the Cur + $NTiO_2$ group compared to those treated with $NTiO_2$ ($p<0.01$).

Table 1. Testis and body weight of the Control and Experimental groups

Groups	Body weight (g)	Testisweight (mg)	Testis/body (mg/g)
Control	30.2±1.7	114.6±13.9	3.81±0.16
Cur	30.3±2.2	115.1±16. 4	3.84±0.21
$NTiO_2$	28.3±1.2	82.1±17.4*	2.9±0.20*
Cur + $NTiO_2$	30.1±1.9	107.4±15.5†	3.56±0.12†

Values are expressed as mean ± SD for 8 mice. *$p<0.05$, $^{\dagger}p<0.05$;
* and † symbols respectively indicate comparison to control and $NTiO_2$-intoxicated groups.

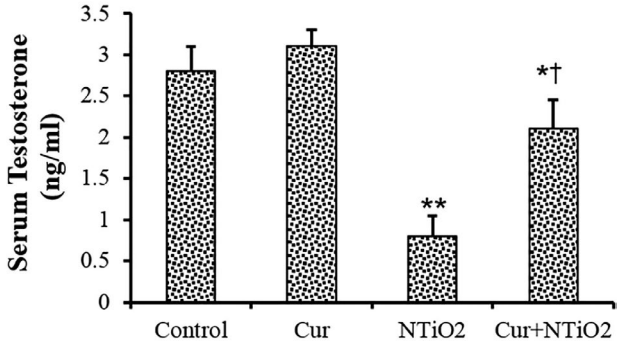

Figure 2. Testosterone assessment of control and experimental groups. *$p<0.05$, **$p<0.01$, $^{\dagger}p<0.01$; * and † symbols respectively indicate comparison to the control and $NTiO_2$-intoxicated groups.

Morphometry

In the Cur-treated mice, the seminiferous tubules' diameter and the seminiferous epithelium height were slightly larger than those in the Control group. The morphometric parameters were significantly decreased in the $NTiO_2$ group ($p<0.01$). In the Cur + $NTiO_2$ treated animals, the morphometric parameters were significantly increased in comparison with the $NTiO_2$-intoxicated mice ($p<0.05$), but no significant alteration in parameters was found when compared to the Control group (Figure 3).

Histology

Testicular sections in both the control and Cur groups had a normal appearance. In the $NTiO_2$ group, disorganization of germ cell layers, detachment, sloughing and atrophy was found, and the histologic criteria were significantly increased ($p<0.01$). Cur administration could attenuate the criteria compared to the $NTiO_2$-intoxicated animals (Table 2 and Figure 4).

Spermatogenesis Assessment

Normal spermatogenesis was see in both control and Cur groups. In the $NTiO_2$ group, abnormal spermatogenesis was found in several tubules of each section and the Johnsen score was significantly decreased when compared to the Control animals ($p<0.01$). In the Cur + $NTiO_2$ group, a few tubules showed maturity arrest and the mean Johnsen score was slightly lower than that in the Control group (Figure 5).

Sperm parameters

The number and motility of sperms in the Cur group were significantly higher than those in the Control were ($p<0.05$), while the abnormality percentage was slightly reduced in this group. $NTiO_2$ induced a significant reduction in all sperm parameters ($p<0.01$). Pretreatment with Cur substantially attenuated the number, abnormality and motility of the sperms in comparison with the $NTiO_2$-intoxicated animals (Table 3).

DISCUSSION

Data from our study showed that the $NTiO_2$ treatment significantly reduced testicular weight, testosterone concentration, morphometric parameters, and

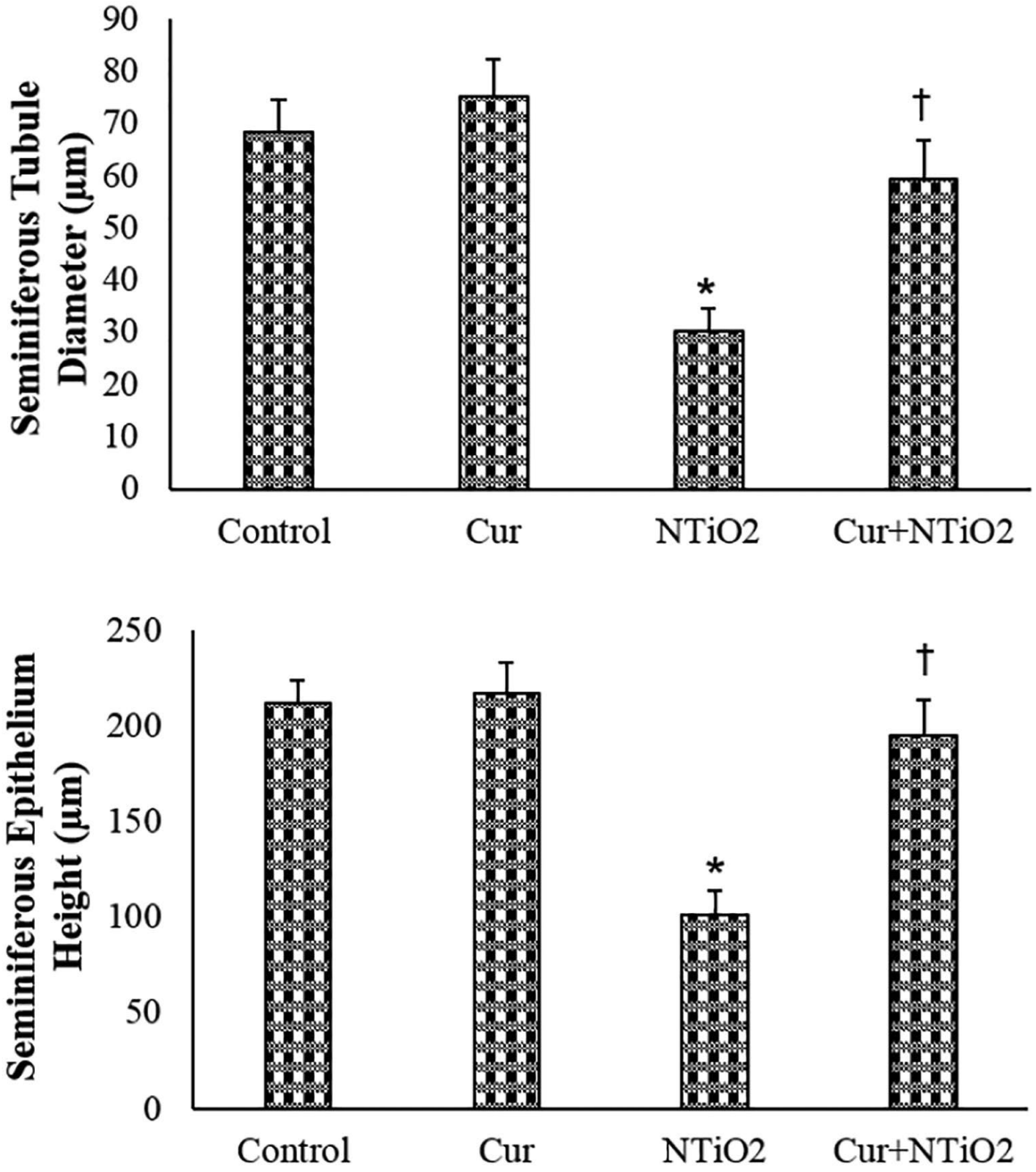

Figure 3. Morphometric parameters of control and experimental groups. *p<0.01, †p<0.05; * and † symbols respectively indicate comparison to the control and $NTiO_2$-intoxicated groups.

Table 2. Histology assessments in the Control and Experimental groups

Groups	Percentage of tubules			
	Normal	Detached	Sloughed	Vacuolated
Control	94.5 ±4.9	1.3±0.5	1.1±0.2	4.6±0.5
Cur	95.7±4.3	1.1±0.2	0.7 ± 0.08	2.5±0.3
$NTiO_2$	45.3±3.6**	23.7±3.1**	18.9±2.3**	42.3±3.1**
Cur+$NTiO_2$	77.6±5.6*†	9.4±2.6*†	8.6±1.4*†	13.1±2.7*†

Values are expressed as mean ± SD for 8 mice. *p<0.05, **p<0.01, †p<0.01;
* and † symbols respectively indicate comparison to control and $NTiO_2$-intoxicated groups.

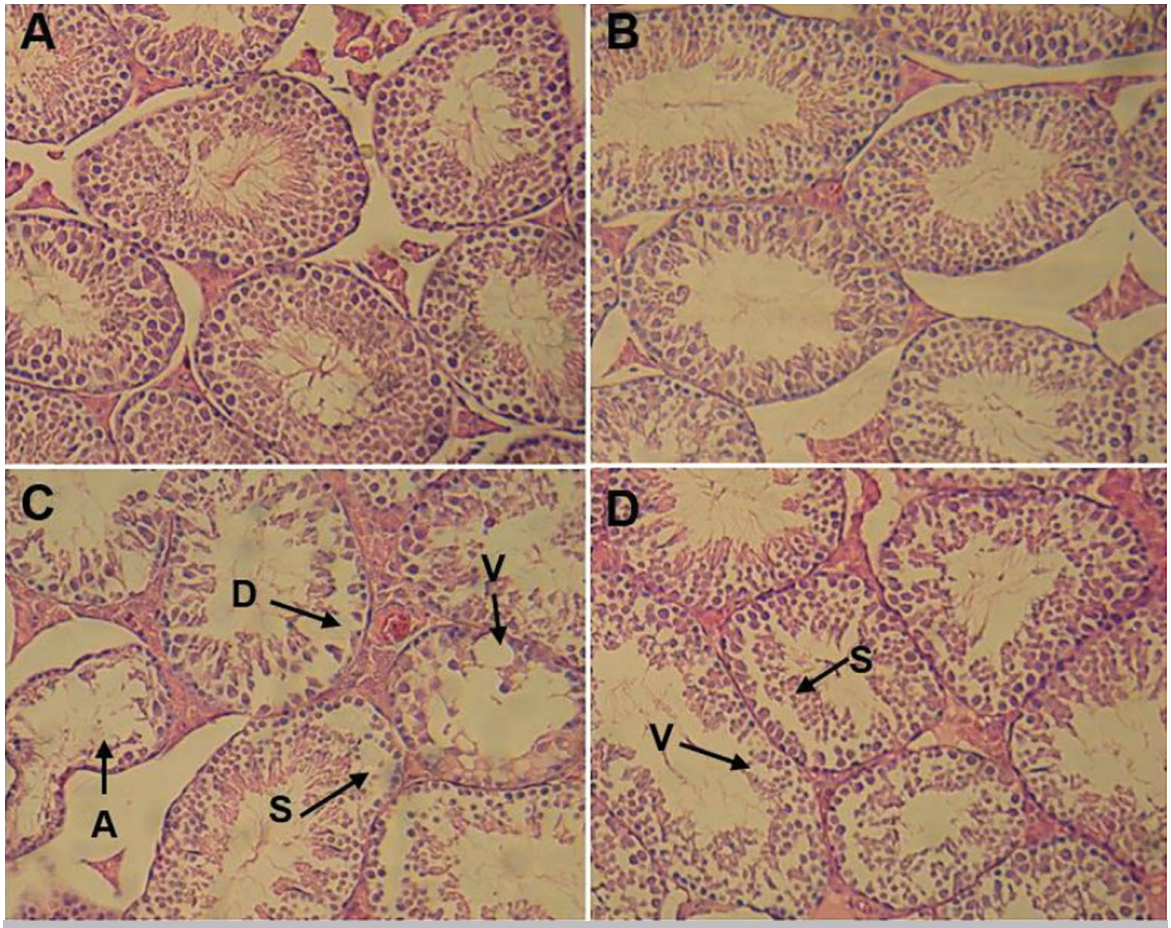

Figure 4. Light microscopy of testicular tissue in various groups (Hematoxylin and eosin staining). Magnifications ×250. A. Control Group; B. Cur group; C. NTiO2-intoxicated group; D. Cur + $NTiO_2$ group. A: atrophy, V: vacuole, S: sloughing, D: detachment.

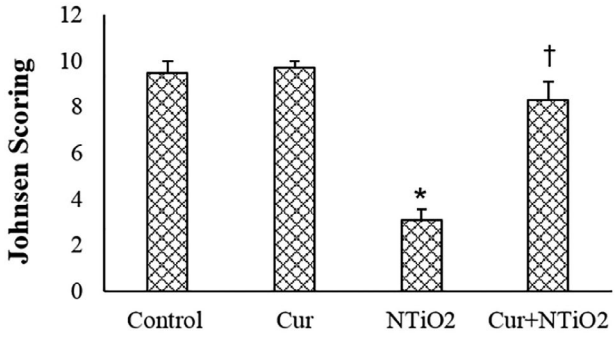

Figure 5. JJohnsen scoring of control and experimental groups. *p<0.01, †p<0.01; * and † symbols respectively indicate comparison to the control and $NTiO_2$-intoxicated groups.

sperm quality. $NTiO_2$ also induced histological changes and maturity arrest. Pretreatment with Cur could effectively attenuate these events.

The reduction in testicular weights of the $NTiO_2$-treated mice indicates the toxic effect of this nanoparticle on mouse testicles. The testicular weight is dependent on the germ cells mass. Thus, the reduction in testicular weight may be a consequence of germ cells death and spermatogenesis defects. In this study, Cur reversed testicular weight loss induced by $NTiO_2$, which is probably caused by the prevention of germ cell death in the seminiferous tubules.

The weight changes after $NTiO_2$ administration were accompanied by alterations in the morphometric parameters. In agreement with our results, Moridian *et al*. (2015) showed that zinc oxide NPs decreased seminiferous tubules' diameter and seminiferous epithelium height. The decrease in seminiferous diameter may indicate germ cell loss induced by $NTiO_2$. The Cur-pretreatment could attenuate morphometric parameters indicated its beneficial effects on male germ cells. Mahmoudi *et al*. (2017) showed that Cur attenuated morphometric parameters of seminiferous tubules in sodium metabisulfite-treated mice. Cur ameliorated morphometric parameters in gentamicin-induced reproductive toxicity (Fetouh & El-Saied Azab, 2014). Ema *et al*. (2010) showed that $NTiO_2$ altered spermatogenesis and induced histological changes in the offspring's' testicles.

The remarkable decrease in morphometric parameters after $NTiO_2$ treatment was accompanied by histological changes to the seminiferous tubules, such as epithelial vacuolization, sloughing, detachment, and atrophy. These histological features are commonly observed in Sertoli cell damage after exposure to various toxicants (Russell & Griswold, 1995). Gao *et al*. (2013) showed that $NTiO_2$ aggregated in the Leydig cells, the Sertoli cells, and spermatids, which led to disruption of seminiferous tubules, reduction on the number of mature sperms, as well as decreased numbers of Sertoli cells in the mice. Zhao *et*

Table 3. Sperm parameters in the Control and Experimental groups				
Parameters	**Control**	**Cur**	**$NTiO_2$**	**$NTiO_2$+ Cur**
Sperm count (106/ml)	47.3±4.4	56.2±4.1*	26.4±4.1**	37.5±8.2*†
Abnormality (%)	23.1±2.4	21.3±2.1	63.7±5.2**	39.6±10.3*†
Rapid progressive (%)	71.3±4.5	79.9±6.3*	23.2±3.1	68.6±4.5†
Slow progressive (%)	15.7±2.6	11.1±1.4	29.1±3.8**	14.9±0.24†
No progressive (%)	7.4±1.4	4.9±1.2	22.3±3.1**	10.8±0.36*†
Immotile (%)	5.6±1.2	3.1±0.9	25.4±2.6**	5.7±0.16†

Values are expressed as mean ± SD for 8 mice. *p<0.05, **p<0.01,†p<0.01.
* and † symbols respectively indicate comparison to the Control and $NTiO_2$-intoxicated groups.

al. (2013) demonstrated that $NTiO_2$ induces apoptosis in Sertoli cells. Hong *et al*. (2016) showed that $NTiO_2$ induced cytotoxicity in primary cultured Sertoli cells of mice.

The Cur pretreatment effectively reversed vacuolization in the germinal epithelium, detachment of germ cells from basal lamina and sloughing of immature germ cells, indicating improvements in Sertoli cell functions. Cur increased the total number of Sertoli cells in gentamicin-induced reproductive toxicity in adult male guinea pigs (Fetouh & El-Saied Azab, 2014). Cur inhibited DNA fragmentation, apoptosis and cell cycle arrest induced by sodium arsenite in cultured murine Sertoli cells (Khan *et al*., 2013). Mahmoudi *et al*. (2017) also reported that Cur prevents structural changes in rat testicles induced by sodium meta-bisulfite.

The results of Johnsen's scoring also showed poor spermatogenesis in $NTiO_2$-treated animals. Alterations in the Johnsen scoring may relate to germ cell degeneration. The decreasing Johnsen score by Cur indicated the preventive effect of Cur on germ cell damage caused by $NTiO_2$.

The administration of $NTiO_2$ significantly reduced testosterone concentrations, which was accompanied by a significant increase in histological criteria. Blanco-Rodríguez & Martínez-García (1998) reported that testosterone withdrawal induced detachment of germ cells from the seminiferous epithelium. Testosterone is required for the attachment of round spermatids to Sertoli cells (Smith & Walker, 2014). Komatsu *et al*. (2008) reported that $NTiO_2$ was absorbed by mouse Leydig TM3 cells and it affected testosterone secretion.

Pretreatment with Cur could raise the testosterone concentration by about 2.6-fold, higher than the $NTiO_2$-intoxicated animals. Cur ameliorated testosterone serum levels in metronidazole-treated mice (Karbalay-Doust & Noorafshan, 2011). Cur protected the Leydig cells of mice against chronic alcohol administration (Giannessi *et al*., 2008). Fetouh & El-Saied Azab (2014) also showed that Cur increased the total number of Leydig cells in gentamicin-treated animals. The improved testicular tissue and increased testicular weights in the Cur-pretreated mice may be the result of restoring testosterone levels.

As shown in the results, $NTiO_2$ induced a significant reduction in sperm count and sperm motility and a significant increase in sperm abnormality. In accordance with our results, Gao *et al*. (2013) reported that the administration of $NTiO_2$ resulted in a decrease in sperm count and motility, increase in sperm abnormalities, and apoptosis of germ cells in rats. In the study by Ema *et al*. (2010), $NTiO_2$ caused a decrease in daily sperm production and a decrease in sperm mobility.

Sperm abnormalities are usually accompanied by sperm inactivation. In $NTiO_2$-intoxicated mice, 26 and 63% of sperms were immobilized and had abnormal morphology, respectively. Our results showed that sperm abnormality in Cur pretreated mice was approximately 1.6 times less than that of the $NTiO_2$-intoxicated animals. Thus, Cur pretreatment may decrease genotoxicity and, consequently, reduce sperm abnormality. Karbalay-Doust & Noorafshan (2011) showed that Cur improved sperm abnormality in metronidazole-treated mice. The group pretreated with Cur also showed a significant increase in the percentage of rapid progressive and slow progressive sperms, and a significant decrease in the percentage of non-progressive and immotile sperms. On the other hand, the number of sperms in the Cur-pretreated groups was approximately 1.4-fold higher than those treated with $NTiO_2$. These findings demonstrate that Cur pretreatment can improve sperm quality. Cur reduced metronidazole-induced testicular toxicity and improved the quality of sperms in mice (Noorafshan *et al*., 2010). Cur improved daily sperm production in gentamicin-induced reproductive toxicity in adult male guinea pigs (Fetouh & El-Saied Azab, 2014). The improved sperm quality in Cur pretreated mice was considered a result of improving testicular histology and elevation of testosterone concentration.

CONCLUSION

This study demonstrated that Cur improved the spermatogenesis defects in $NTiO_2$-treated mice. This study suggests that Cur can protect germ cells by enhancing testosterone secretion. Further experiments are needed to clarify the mechanisms of the Cur effect on NP-induced toxicity. This study was conducted on mice and cannot be recommended for human clinical use. However, Cur has been suggested as a possible treatment for male reproductive disorders in humans exposed to high doses of nanoparticles.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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