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The therapeutic effect of cerium oxide nanoparticle on ischaemia/reperfusion injury in rat testis

Ali Mousavi[1] | Ahmad Gharzi[1] | Mohammadreza Gholami[2] | Fatemeh Beyranvand[3] | Mohsen Takesh[1]

[1]Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

[2]Department of Anatomical Sciences, Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

[3]Department of Surgery, Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran

Correspondence
Mohsen Takesh, Department of Biology, Faculty of Science, Razi University, Kermanshah 6714967346, Iran.
Email: mohsen.takesh@gmail.com

Abstract

Testicular torsion is a dangerous urogenital disorder which is caused by twisting of spermatic cord, and unless immediate treatments happen at a proper time, oxidative stress, occurred during ischaemia reperfusion, finally leads to irreversible disintegration of testicular tissue. One of the first preventive lines is to administrate antioxidant factors. In the present study, we investigate the therapeutic effect of cerium oxide nanoparticle on the injury. We divided 45 rats into nine groups, subjected eight groups to testicular torsion–detorsion, injected different doses of cerium oxide nanoparticle into the peritoneum of six groups and analysed all the groups regarding spermatogenetic indices including sperm count, sperm viability and Johnson mean. Our results showed that cerium oxide nanoparticle can alleviate oxidative stress in testis, and this alleviation promotes the reproductive indices as the concentration of cerium oxide nanoparticles increases. The catalase-mimetic and superoxide dismutase-mimetic activities of cerium oxide nanoparticle are the most probable theories to explain the antioxidant effect of the nanoparticle.

KEYWORDS

detorsion, orchiectomy, oxidative stress, torsion

1 | INTRODUCTION

Testicular torsion (TT) is an acute progressive urogenital disorder, which is caused by twisting of spermatic cord (Sharp et al., 2013). Aetiologically, the disease would be categorised into extravaginal type, which occurs during perinatal life as the tunica vaginalis is not appropriately stabilised to the inner scrotal layers, and intravaginal type, which occurs during pubertal or adolescent life as the stabilising is located more proximally to spermatic cord permitting the suspended testicle to twist within tunica vaginalis (Fehér & Bajory, 2016; Tournaye et al., 2017). The main symptoms of the intravaginal type usually are severe unilateral scrotal pain, nausea and vomiting, and physical examination may reveal a high-riding testicle with an absent cremasteric reflex (Fehér & Bajory, 2016). The twisting initially escalates venous pressure and congestion, and then, arterial blood decreases and ischaemia occurs (Nelson et al., 2016). In this situation, the patient has just four to eight hours to protect himself from permanent ischaemic testicular damage and to release the torsion (i.e. detorsion); otherwise, such a condition might lead to permanent decreased fertility or might necessitate orchiectomy (Kapoor, 2008). On the other hand, even if the detorsion occurs at an appropriate time, it might induce reperfusion injury, which causes further damage to the ischaemic testis through generating reactive oxygen species (ROS), proinflammatory cytokines, neutrophil recruitment, lipid peroxidation, anoxia and apoptosis (Hazeltine & Panza, 2017).

Over the past decades, numerous studies have investigated the effects of different strategies and drugs on controlling testicular ischaemia–reperfusion (IR) injury (Dokmeci, 2006; Hazeltine & Panza, 2017; Turner & Lysiak, 2008). Except scrotum cooling, however, no other treatment has successfully alleviated the oxidative stress in clinical practice (Boyarsky, 1989). Although its

detailed pathophysiological mechanism remains unclear (Fitridge & Thompson, 2011), ROS and numerous toxic substances, generated meanwhile, play important roles in the pathogenesis (Inbaraj & Chen, 2020). In this regard, exploring the agents that protect testis against destructive factors would be an inevitable essence.

Numerous studies showed the antioxidant, anti-inflammatory and antiangiogenic effects of cerium oxide nanoparticle (CeO[NP]) on alleviating oxidative stress in many pathological disorders (Nelson et al., 2016; Serebrovska et al., 2017). The most explicit effects in the disorders are anti-cancer effects (Hijaz et al., 2016), Alzheimer's disease (Kim et al., 2019), amyotrophic lateral sclerosis (DeCoteau et al., 2016), anti-obesity (Rocca et al., 2015), anti- inflammation (Oró et al., 2016), hepatic ischaemia reperfusion (Manne et al., 2017), constipation (Yefimenko et al., 2015), sepsis and acute hepatic/ kidney injury (Manne, Arvapalli, Nepal, Shokuhfar, et al., 2015; Manne, Arvapalli, Nepal, Thulluri, et al., 2015), radiation-induced tissue damage (Das et al., 2018) and retinal disease (Fiorani et al., 2015). Nonetheless, studies on male reproductive system demonstrated incompatible results; some studies declare that it has toxic, disturbing or disrupting impacts on the normal activity and function of the system (Adebayo et al., 2018; Hamzeh et al., 2018), whereas the others show protective and antioxidative effects on the system (Artimani et al., 2018; Hamzeh et al., 2019; Saleh et al., 2020); the adverse effects of CeO2 might be dose dependent.

Although CeO[NP] can present potential bioactivities, novel strategies to improve their pharmacokinetics and targeting capabilities are still needed to be explored. In the present study, we investigated the effect of CeO[NP] on male reproductive system following IR injury through analysing the count, viability and morphology of spermatozoa.

2 | MATERIAL AND METHODS

2.1 | Experimental animals and housing conditions

We conducted an experimental study on 45 adult male rats (race = wistar; age = 8 weeks old; mean weight = 200 ± 20 g). We purchased them from Razi herbal medicines research centre, Khorramabad, Lorestan. All the animal procedures were approved by the Ethical Committee of Razi University, Kermanshah, Iran. We housed the specimens in a temperature-controlled room (21–25°C) having a 12-hr:12-hr photoperiod condition with ad libitum to food and water.

2.2 | Experimental design

We randomly divided the specimens into nine groups. The first was healthy group (G0), which was subjected to no injection and no surgical procedure. The second was served as a control group (GC) submitted to torsion–detorsion procedure. The third, as a negative control group (GNC), was just injected by 4 ml distilled water. For the other groups, following subjecting to torsion–detorsion procedure, we intraperitoneally injected 4 U CeO[NP] with the following concentrations, that is 1, 2, 5, 10, 20 and 50 mg/kg, to the groups I–VI (GI–GVI) respectively (Table 1). We repeated all the injections weekly at the same time the same as the first one through six subsequent weeks.

TABLE 1 A summary of the designed experiment to explore the effect of CeO[NP] on IR injury

Groups	Symbol	Treatments
Healthy	G0	None
Control	GC	Ischaemia + Reperfusion
Negative control	GNC	Ischaemia + Reperfusion + injecting 4 ml distilled water
Group 1	GI	Ischaemia + Reperfusion + injecting 1 mg/kg (4 U)
Group 2	GII	Ischaemia + Reperfusion + injecting 2 mg/kg (4 U)
Group 3	GIII	Ischaemia + Reperfusion + injecting 5 mg/kg (4 U)
Group 4	GIV	Ischaemia + Reperfusion + injecting 10 mg/kg (4 U)
Group 5	GV	Ischaemia + Reperfusion + injecting 20 mg/kg (4 U)
Group 6	GVI	Ischaemia + Reperfusion + injecting 50 mg/kg (4 U)

2.3 | Surgical procedure

We anaesthetised all the specimens using an intraperitoneally one-shot injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). We shaved the scrotal skin of the specimens and disinfected by 10% povidone–iodine solution and incised the skin on left testis to access it. Then, we created torsion through 720°-counterclockwise twisting and maintained by fixing the testis to scrotum by a 5-0 nylon suture passing through tunica albuginea and dartos. The testis was left on the top of the incised region, covered with a sterile gauze pad and kept moist with normal saline, while the rat was anaesthetised. After 45 min, we removed the suture and untwisted the testis to restore in scrotum for reperfusion. We stitched the incision in two layers and rubbed tetracycline ointment to disinfect. We nourished the rats for 6 weeks. We anaesthetised and euthanised all the specimens just at the beginning of the seventh week using intracardiac potassium chloride injection. Then, we orchiectomised the left testis and fixed it in Bouin's fluid (Fox et al., 1985).

2.4 | Analytical procedure

We quickly removed the tail of the epididymis and put it into 5.0 ml Ham's F-10. We chopped it with a sharp scissor, incubated it for 5 min at 37°C in an atmosphere of 5% CO_2 and let the spermatozoa get out and float in the media. The sperm suspension remained

in the incubator (37°C) for 20 min: (1) We diluted 20 μl aliquot of the suspension with the same volume of nigrosine–eosin (1% eosin Y and 5% nigrosine). Then, we prepared a smear of the content, and after drying, we evaluated the smear using a light microscope (BX51P, Olympus) at 400× magnification. Live spermatozoa are not being stained, whereas the dead ones seem coloured. We counted 200 spermatozoa by observing the proportion of unstained spermatozoa to the coloured. We expressed the viability as percentage of live spermatozoa (viable) on total spermatozoa counted (Badkoobeh et al., 2013; Organization, 2010); (2) we spread 5 μl aliquot of the suspension on a glass slide and let dry and fixed the smears using 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. We stained the slides using 5% aqueous aniline blue mixed with 4% acetic acid (pH 3.5) for 5 min and evaluated 200 spermatozoa per slide at 400× magnification regarding morphological abnormalities for head, middle piece and tail (Hammadeh et al., 1996; Organization, 2010). The results were expressed as percentage of normal cells on total spermatozoa counted; and (3) we diluted 5 μl aliquot of the suspension with 95 μl of a diluent (0.35% formalin containing 5% $NaHCO_3$ and 0.25% trypan blue) and transferred approximately 10 μl of this diluted specimen on a haemocytometer standing for 5 min until the spermatozoa deposited (Organization, 2010). Then, we counted spermatozoa at 400× magnification.

We embedded the fixed testis in paraffin longitudinally and sectioned the tissue block in 5 μm thickness. We stained the sections with haematoxylin and eosin (HE) for histopathological examination (Fischer et al., 2008). We evaluated the stained slides at 400× magnification regarding modified Johnson scoring to investigate the maturation and quality of seminiferous tubules (Holstein et al., 2003), as noted in Table 2.

2.5 | Statistical analysis

All data were expressed as mean and standard deviation. We statistically analysed the data using SPSS (version 19.0, IBM). Parametric group data were compared using ANOVA with Tukey's post hoc test and Student's *t* test. Data were statistically significant at $p < .05$.

3 | RESULTS

Our results showed that IR injury decreases considerably the variables including sperm count and viability as well as Johnson mean, as represented in the control and negative control groups related to the healthy group (Tables 3 and 4). Histological microscopy confirmed it as per the seminiferous tubule of the healthy group, wherein the spermatogonia align in two or three continuous layers and various types of germ cells exist between the basal lamina and the sperm-filled lumen (Figure 1a), seminiferous epithelia in the control and negative control groups are significantly reduced, and no haploid cells exist in their lumens; spermatogonia and primary spermatocytes exist across the epithelium (Figure 1b,c).

Treatment with 1 mg/kg CeO[NP] in group 1 showed a substantial increase in sperm count related to the negative control group, but all the other variables were not substantially changed related to the control and negative control group (Tables 3 and 4). Microscopically, group 1 resembles the negative control group regarding the luminal shape irregularity, occurrence of meiosis 1, existence of spermatogonia and primary spermatocytes, and absence of haploid cells (Figure 1d);

Treatment with 2 mg/kg CeO[NP] in group 2 showed a substantial increase in sperm count related to the negative control group and viability and Johnson mean related to the control and negative control groups; as well, the viability was considerably increased related to group 1 (Tables 3 and 4). Microscopically, group 2 would be roughly the same as group 1 but the lumen dwindles in diameter (Figure 1e).

Unless sperm count related to the control group, 5 mg/kg CeO[NP] in group 3 increased substantially all the variables related to the control and negative control groups; as well, viability and Johnson mean were substantially increased related to groups 1 and 2 (Tables 3 and 4). Microscopically, group 3 would be characterised

TABLE 2 Tabulated guidance of modified Johnson index about the maturation and quality of seminiferous tubules

Score	Qualification
10	Presence of full spermatogenesis present
9	Incomplete spermatogenesis with many late spermatids
8	Presence of <5 spermatozoa per tubules and a few late spermatids
7	Presence of many early spermatids but absence of spermatozoa or late spermatids
6	Presence of few early spermatids but absence of spermatozoa or late spermatids
5	Presence of many spermatocytes but absence of spermatozoa or spermatids
4	Presence of few spermatocytes but absence of spermatozoa or spermatids
3	only Presence of spermatogonia
2	Only presence of Sertoli cells and absence of germinal epithelial cells
1	Absence of seminiferous epithelium exists

TABLE 3 A comparison of sperm parameters and Johnson's score (mean ± standard deviation) between different groups during exploring the effect of CeO[NP] on IR injury

Groups	*n*	Sperm counting ($\times 10^{-6}$)	Sperm viability	Johnson mean
G0	5	54.00 ± 2.04	94.70 ± 1.50	10.00 ± 0.00
GC	5	12.12 ± 0.90	67.50 ± 1.30	3.80 ± 1.20
GC$^-$	5	17.40 ± 1.73	68.00 ± 0.90	4.00 ± 1.10
GI	5	6.92 ± 0.45	69.70 ± 2.30	4.20 ± 1.40
GII	5	8.88 ± 0.82	72.75 ± 2.20	4.60 ± 1.50
GIII	5	8.80 ± 1.19	77.50 ± 2.10	5.20 ± 1.80
GIV	5	19.44 ± 2.15	77.90 ± 1.20	6.00 ± 1.80
GV	5	27.16 ± 4.95	81.60 ± 1.10	6.60 ± 1.80
GVI	5	28.69 ± 3.63	85.80 ± 0.80	7.30 ± 1.60

by an irregular lumen, resumption of meiosis 1 and 2, and high presence of spermatogonia, primary spermatocytes and secondary spermatocytes but absence of spermatozoa and spermatids (Figure 1f).

All except sperm count were substantially changed related to the control and negative control groups following treatment with 10 mg/kg CeO[NP] in group 4; all the variables, however, were substantially changed related to groups 1–3 (Tables 3 and 4). Histological microscopy in group 4 shows that the lumen is regular in shape; the germ layer becomes more thickened; meiosis 1 and 2 resume; spermatogonia, primary spermatocytes and secondary spermatocytes exist frequently, and some spermatids are generated (Figure 1g).

20 mg/kg CeO[NP] in group 5 increased considerably all the variables related to the control and negative control groups; moreover, all except the viability compared to group 4 were considerably changed related to groups 1–4 (Tables 3 and 4). In group 5, histological microscopy shows that the lumen sounds regular, seminiferous epithelium thickens further, plenty of spermatids exist, and few spermatozoa are produced (Figure 1h).

50 mg/kg CeO[NP] in group 6 enhanced all the variables related to other groups except the viability of group 5. Group 6 shows a regular lumen filled with some spermatozoa. The thickened seminiferous epithelium generates frequent spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids (Figure 1i).

4 | DISCUSSION

The pathogenical aetiologies included in testicular IR injury, summarised by Karaguzel et al. (2014), might be considered as the following summary: neutrophil recruitment, generation of ROS, proinflammatory cytokines and adhesion molecules, lipid peroxidation, apoptosis, anoxia and alteration to microvascular blood flow. As a higher category, the oxidative stress refers to a condition through which the production of ROS, including peroxynitrite, peroxide, hydroxyl radicals, and superoxide anions, surpasses its deletion (Huang et al., 2016). The most important consequences of IR in testicular cells are the aggregations of hypoxanthine and xanthine from the metabolism of ATP and the substantial conversion of xanthine dehydrogenase to xanthine oxidase a proteolytic enzyme which damages the cytoskeleton and membrane of testicular cells and generates superoxide (Aitken & Roman, 2008; Reilly et al., 1991). Of the consequences of the short-term exposure (≤3 hr), Guimarães et al. (2007) mentioned the testicular glutathione depletion and spermatogenesis disruption as well as increased testicular peroxidative harms that occurs following detorsion. Once the reperfusion occurs, the oxygen accessed for aerobic metabolism can produce ROS (Üstün et al., 2008), which changes membrane permeability and disrupts cell integrity through peroxiding cellular and mitochondrial membranous lipids (Su et al., 2019). Probably like myocardial infarction, the oxidation of essential -SH groups and/or a limited proteolytic clip in the injury convert xanthine dehydrogenase to xanthine oxidase. Once the tissue is reperfused with blood, xanthine oxidase decomposes oxidisable substrate in the form of xanthine/hypoxanthine and produces enormous ROS. The sudden induction of lipid peroxidation and the concomitant suppression of endogenous antioxidant activities including superoxide dismutase, catalase and glutathione peroxidase accompany the damage (Avlan et al., 2005; Ünsal et al., 2006).

In the present study, the histopathological changes of the testicular tissue due to torsion and detorsion illustrates the effect of ischaemia in a designated period of 45 min, which would be evident from the score (Table 3) and the cross-sectional view of Photomicrograph (Figure 1b,c). At this step, seminiferous tubules show ceased spermatogenesis, and mainly spermatogonia and primary spermatocytes might be observed in the cross section. Elshaari et al. (2012), however, showed that by a designated period of 30 min, seminiferous tubules illustrate normal spermatogenesis and all stages of spermatogenic cells including abundant spermatozoa, even though some sloughing and tissue disorganisation are observable; it might be due to the initiation of degenerative process as a result of increased lipid peroxidation and the cellular ATP depletion. A designated period of 1 hr, however, caused to mildly affected spermatogenesis with severe sloughing and tissue disorganisation (Elshaari et al., 2012). Comparatively, this description might support the histopathological changes in our study during the period of 45 min.

Due to the regenerative antioxidant characteristic, numerous studies have already investigated the protective effect of CeO[NP] on oxidative damage in testis (Moridi et al., 2018; Saleh et al., 2020). In the endothelial injury of cardiovascular diseases caused by oxidative stress, CeO[NP] can prevent the detrimental effect of H_2O_2 and attenuate ROS overproduction induced by H_2O_2. It also can alleviate apoptosis. CeO[NP] possesses two main advantages: (a) due to small size, it owns a high surface area to volume ratio; (b) due to existence in either Ce^{3+} or Ce^{4+} states on the particle surface, it allows the formation of surface oxygen vacancies (Naganuma & Traversa, 2012). The coexistence of Ce^{3+} and Ce^{4+} ions generates oxidoreduction reactions eliminating ROS in catalase-mimetic and superoxide dismutase-mimetic activities: oxidising Ce^{3+} to Ce^{4+} causes superoxide to be reduced to H_2O_2; reduction of Ce^{4+} to Ce^{3+} causes H_2O_2 to be oxidised to O_2 (Singh & Singh, 2019). As Patel et al. mentioned, a significant increase

TABLE 4 Statistical significance of intergroup comparison during exploring the effect of CeO[NP] on IR injury; + = significant, − = insignificant

Groups	variables	G0	GC	GC⁻	GI	GII	GIII	GIV	GV	GVI
G0	Sperm count	0								
	Sperm viability	0								
	Johnson mean	0								
GC	Sperm count	+	0							
	Sperm viability	+	0							
	Johnson mean	+	0							
GC⁻	Sperm count	+	−	0						
	Sperm viability	+	−	0						
	Johnson mean	+	−	0						
GI	Sperm count	+	−	+	0					
	Sperm viability	+	−	−	0					
	Johnson mean	+	−	−	0					
GII	Sperm count	+	−	+	−	0				
	Sperm viability	+	+	+	+	0				
	Johnson mean	+	+	+	−	0				
GIII	Sperm count	+	−	+	−	−	0			
	Sperm viability	+	+	+	+	+	0			
	Johnson mean	+	+	+	+	+	0			
GIV	Sperm count	+	−	−	+	+	+	0		
	Sperm viability	+	+	+	+	+	+	0		
	Johnson mean	+	+	+	+	+	+	0		
GV	Sperm count	+	+	+	+	+	+	+	0	
	Sperm viability	+	+	+	+	+	+	−	0	
	Johnson mean	+	+	+	+	+	+	+	0	
GVI	Sperm count	+	+	+	+	+	+	+	−	0
	Sperm viability	-	+	+	+	+	+	+	+	0
	Johnson mean	+	+	+	+	+	+	+	+	0

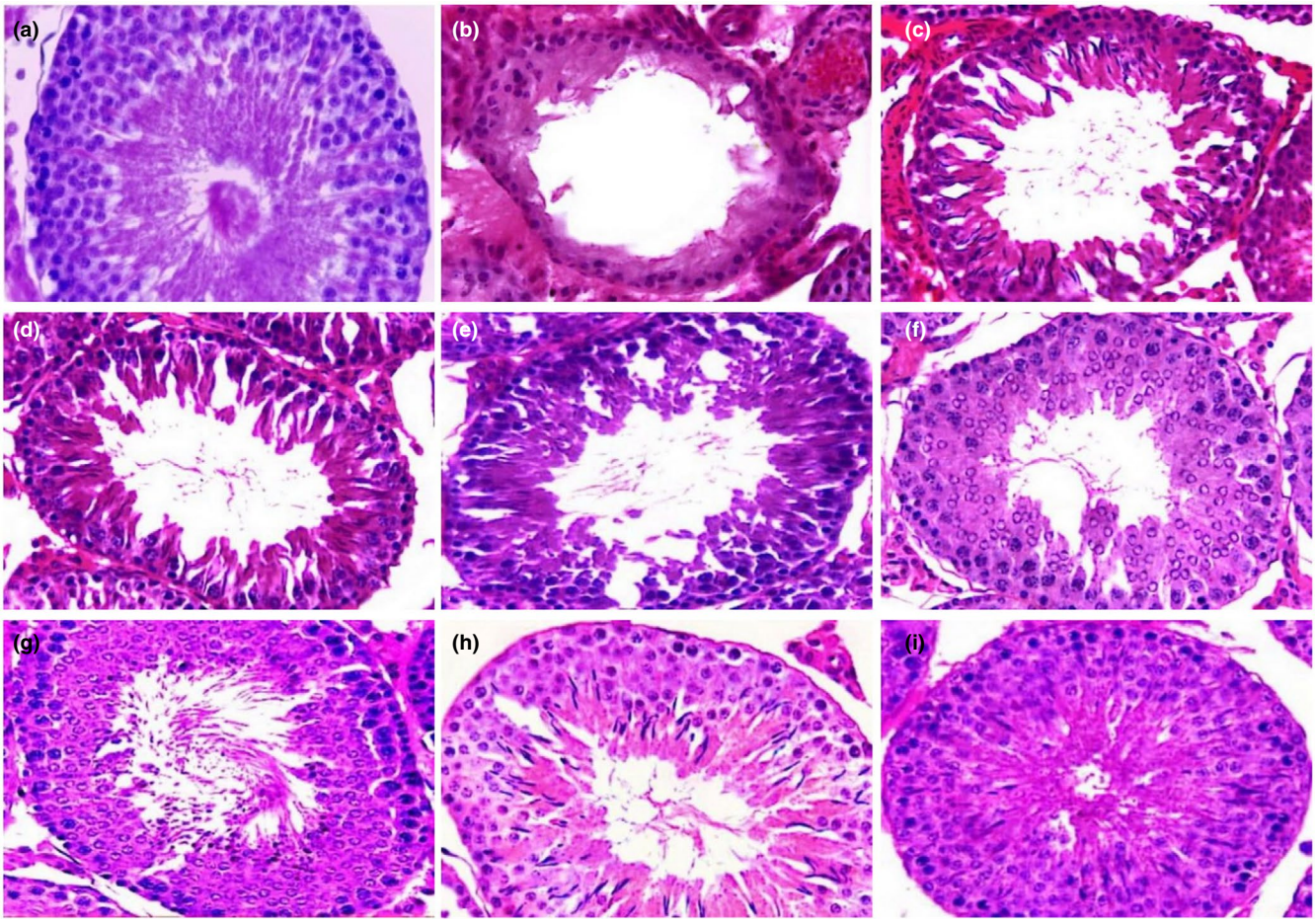

FIGURE 1 Sectional views of seminiferous tubules; (a) healthy group, (b) control group, (c) negative control group, (d) group 1, (e) group 2, (f) group 3, (g) group 4, (h) group 5, (i) group 6. Stain: Haematoxylin–eosin (H–E) staining

occurs in the cellular uptake and internalisation of CeO [NP] in a concentration-dependent (10–100 μg/ml) manner, so that CeO[NP] significantly reduces the amount of ROS (Patel et al., 2018). This might explain our results, wherein the spermatogenesis indices improved as the concentration of CeO [NP] increased (Tables 3 and 4). The effect of increased concentrations of CeO[NP] on sperm parameters (count, motility and viability) is evident in Moridi et al., so that they concluded that total antioxidant capacity of testis cells enhances (Moridi et al., 2018).

In conclusion, the present study showed that CeO[NP] can alleviate the effect of oxidative stress caused by IR injury in testis. The indices representing normal spermatogenesis including sperm count and viability as well as Johnson mean were promoted following dosage leverage, so that the best results were achieved at 50 mg/kg. Despite contradicting reports, further studies might reveal its therapeutic nature and decipher its physiological and biochemical mechanisms in the stress.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ali Mousavi https://orcid.org/0000-0002-1697-2651
Ahmad Gharzi https://orcid.org/0000-0001-8373-0487
Mohammadreza Gholami https://orcid.org/0000-0003-3719-1659
Mohsen Takesh https://orcid.org/0000-0002-0990-1126

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