HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.08.016

Role of zinc as an antioxidant and anti-inflammatory to relieve cadmium oxidative stress induced testicular damage in rats

Samir Abd El-Monem Bashandy[1], Enayat Abdel Aziz Omara[2], Hossam Ebaid[3,4], Mohamed Mahmoud Amin[1*], Mahmoud Sanad Soliman[5]

[1]*Department of Pharmacology, Medical Division, National Research Centre, Dokki, Giza, Egypt*

[2]*Department of Pathology, National Research Centre, Dokki, Giza, Egypt*

[3]*Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia*

[4]*Department of Zoology, Faculty of Science, El-Minia University, El-Minia, Egypt*

[5]*Department of Therapeutic Chemistry, National Research Centre, Dokki, Giza, Egypt*

ARTICLE INFO

Article history:
Received 18 Apr 2016
Received in revised form 16 May, 2nd revised form 3 Jun, 3rd revised form 16 Jun 2016
Accepted 22 Aug 2016
Available online 30 Sep 2016

Keywords:
Zinc
Cadmium
Oxidative stress
Immunohistochemistry
Sperms
Inflammatory markers

ABSTRACT

Objective: To investigate the role of zinc in reducing the deleterious effects of cadmium on male gonads.

Methods: Rats were injected subcutaneously with $CdCl_2$ and $ZnCl_2$ at dose level of 2.2 mg/kg (1/40 of LD_{50} of cadmium per day).

Results: The rats treated with cadmium exhibited a significant increase in levels of testicular malondialdehyde, tumor necrosis factor-alpha, nitrogen oxide and inducible nitrogen oxide synthase immunostaining reaction, as well as an elevation of blood hydroperoxide and follicle stimulating hormone. In addition, a significant decrease in testicular ascorbic acid, zinc, reduced glutathione, catalase, superoxide dismutase, sex organ weight, plasma testosterone and luteinizing hormone were observed in the cadmium group. Sperm motility and count were decreased with cadmium treatment, while sperm abnormalities elevated significantly. Zinc treatment was found to mitigate the toxic effects of cadmium on oxidative stress, spermatogenesis, sex hormones, and inflammatory markers. Rats injected with cadmium showed intense histopathological changes. Zinc manifested protective role and markedly reduced tissues damage induced by cadmium.

Conclusions: The protective effect of zinc can be attributed to its antioxidant and anti-inflammatory properties.

1. Introduction

Zinc is an essential trace element. Zinc affects vital processes including cell proliferation, immune function and defense against free radicals [1–3]. It activates antioxidant system that prevents cell damage [4,5].

Zinc is incorporated in oxidant defense system and functions at many levels [6]. The antioxidant property of zinc is thought to be through maintaining an adequate level of metallothionein and it is essential component of Cu/Zn superoxide dismutase (SOD) [7,8].

Supplement of zinc to protein deficient animals reduced lipid peroxidation (LPO) and enhanced the levels of reduced glutathione (GSH) and SOD activity [9]. Furthermore, elevated LPO was observed in different rat tissues reduced by zinc treatment [10,11]. In addition, zinc appears to collaborate in DNA repair [8]. It has efficiency role in cell division and differentiation [7]. It is reported that zinc promotes spermatogenesis and affects sperm motility [12].

*Corresponding author: Mohamed Mahmoud Amin, Department of Pharmacology, Medical Division, National Research Centre, 33 EL Bohouth St., P.O. Box 12622, Dokki, Giza, Egypt.
Tel: +20 1010000560
E-mail: mohamedmahmoud1983@gmail.com

All experimental procedures involving animals were conducted in accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by Animal Care and Use of King Saud University in Saudi Arabia.

Foundation Project: Supported by King Saud University, Deanship of Scientific Research, College of Science Research Centre.

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

It has been shown that zinc deficiency is related to a reduction in testicular volume, and in serum concentration of testosterone as well as increased levels of serum malondialdehyde (MDA), tissue tumor necrosis factor-alpha (TNF-α), and apoptosis of the germ cells [13]. Zinc interacts with cadmium metabolism through induction of the synthesis of metallothionein [14,15]. Otherwise, zinc deficiency reinforces cadmium toxicity.

The sources of cadmium pollution were electroplating, pigment, plastic, fertilizer industries, and cigarette smoke [16]. It appears that the free radical production is a mechanism of cadmium toxicity that causes pathological conditions in humans and animals [17]. Cadmium causes animal and human reproductive toxicity [18,19].

Cadmium has been related to haemorrhagic necrosis of testicular tissues [20] and an increase in the percentage of abnormal sperms [21]. Cadmium exposure lowered testicular zinc [22] and raised the iron [23] that result in an increase of oxidative stress and testicular damage.

The present study was planned to show whether subcutaneous administration of zinc can ameliorate the toxic effects of cadmium, in terms of (i) recovery in sex hormones levels, indications of testicular oxidative stress and inflammatory markers, (ii) its effect on testicular cadmium, zinc and ascorbic acid, (iii) its effect on sperm parameters, the histology of the testis and immunohistochemistry.

2. Materials and methods

2.1. Chemicals

$CdCl_2$, $ZnCl_2$, 2,4-dinitrophenylhydrazine (DNPH) and thiourea were bought from Sigma Chemical Co. (St. Louis MO, USA). All other chemicals used were of analytical grade.

2.2. Animals

Ninety-six male Wistar albino rats, weighting 180–200 g, were selected from the animal house in Faculty of Pharmacy, King Saud University. Each six animals were kept in a polypropylene cage and left to naturalize laboratory environment. Temperature [(23 ± 1) °C], humidity (50% ± 15%) and normal photoperiod (12–12 h light–dark cycles) were adjusted. The animals were fed dry ration in form of pellets.

2.3. Ethics statement

This experiment was carried out in according to recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 85–23, revised 1996) and under regulations of Animal Care and Use of King Saud University in Saudi Arabia. All the surgeries were performed under deep sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

2.4. Experimental design

The rats were sorted into four equal groups, each containing 24 rats. Group I served as control group. Rats in Group II were injected subcutaneously with $CdCl_2$ at dose level of 2.2 mg/kg (1/40 of LD_{50} of cadmium per day) [24]. Rats in Group III were injected subcutaneously with $ZnCl_2$ at dose level of 2.2 mg/kg [25], followed by $CdCl_2$ injection as described previously. Rats in Group IV were injected subcutaneously with $ZnCl_2$ at dose level of 2.2 mg/kg [25].

The treatments were carried out four times weekly for 2 months. The samples were collected every 2 weeks and six rats from each group were sacrificed. All the parameters were evaluated at four time intervals except testicular catalase (CAT), SOD, GSH, ascorbic acid, TNF-α and nitrogen oxide (NO) which determined at the end of experiment. Also, histology and immunohistochemistry were investigated at 8th week.

2.5. Sperm analysis

Right epididymis was clipped into little pieces with razor blade and scattered in 3 mL of phosphate buffered saline (PBS) (pH 7.2) that kept in an incubator at 37 °C. About 20 μL of above mixture was immediately put on dry, clean and warm slide and examined (400×) under binocular microscope with warmed stage and about 300 spermatozoa were evaluated. Motility was then expressed as the percentage of motile spermatozoa.

The suspension was filtered. The filtrate (up to 0.5 mL) was drawn in leukocyte hemocytometer and diluted with PBS up to the mark 11. The suspension was well-mixed and charged into Neubauer's counting chamber. The total sperm count in 8 big squares was determined to express the number of spermatozoa/epididymis (million/epididymis) [26].

The filtrate was stained with 1% eosin and morphological defects were analyzed under light microscope (40×) and any abnormalities of either heads or tails were noted. Three hundred sperms were screened investigated for each animal and total abnormality was expressed as percentage.

2.6. Determination of testicular cadmium and zinc

Testicular cadmium or zinc concentration (μg/g dry tissue) was affirmed in testes by use of atomic absorption, Model RA-2 (Tokyo, Japan). Samples of testes were put in oven and dried. Certain weight of dried samples were heated with 7 mL nitric acid and per chloric acid (2:1, v/v) in boiling water bath until evaporation. After digestion, the samples were diluted by definite volume of distilled water and filtered.

2.7. Biochemical analysis

Plasma testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations were measured determined by enzyme immunoassay using commercial kit from Diagnostic Products Co. (Los Angeles, CA, USA). Also, the level of TNF-α in testicular homogenates was assayed by ELISA (R&D Systems, Minneapolis, MN USA). The testicular level of NO was evaluated using colorimetric assay kit following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). Blood hydroperoxide level was determined colorimetrically using an analytical system (Iram, Parma, Italy). This method depends on reaction of buffered chromogenic substance with free radicals generated by hydroperoxide. MDA, GSH, CAT and SOD levels of testicular homogenates were determined using colorimetric assay kits (Biodiagnostic, Giza, Egypt). Testicular vitamin C was determined using DNPH [27]. To 0.5 mL of supernatant, 0.5 mL of DNPH reagent (2%

DNPH and 4% thiourea in 9 mol/L sulfuric acid) was added and incubated for 3 h at room temperature. After incubation, 2.5 mL of 85% sulfuric acid was added and color developed was read at 530 nm after 30 min.

2.8. Histological studies

At the end of the experiment, testes from each sacrificed rat were dissected. Then, they were fixed in 10% buffered formalin and were processed for paraffin sectioning by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks. Sections of about 5 μm thickness were stained with Harris hematoxylin and eosin for histological study.

2.9. Immunohistochemical analysis

Deparaffinized slices were incubated overnight with the antibodies against inducible nitrogen oxide synthase (iNOS) diluted 1:100. Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody detection DAKO EnVision+ System, Peroxidase/DAB kit was employed. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted. The immunostaining intensity and cellular localization iNOS, was analyzed by light microscopy.

2.10. Statistical analysis

Data were presented as means ± SEM, and analyzed by One-way ANOVA followed by least significant difference using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cadmium and zinc testicular concentration

Table 1 shows that zinc treatment of cadmium intoxicated rats significantly lowered the accumulation of cadmium in testicular tissue at different time intervals. Moreover, the testicular zinc level of the zinc and cadmium group in combination was significantly higher than that of the cadmium group after 4, 6 and 8 weeks.

Table 1
Testicular zinc and cadmium of treated groups.

Group	Testicular cadmium (μg/g)				Testicular zinc (μg/g)			
	Week 2	Week 4	Week 6	Week 8	Week 2	Week 4	Week 6	Week 8
Group I	0.26 ± 0.07	0.19 ± 0.04	0.22 ± 0.06	0.20 ± 0.02	122.85 ± 8.63	119.14 ± 7.41	110.62 ± 5.42	125.42 ± 9.33
Group II	17.50 ± 0.58[a]	26.26 ± 0.85[a]	44.65 ± 1.07[a]	93.78 ± 2.14[a]	128.22 ± 11.35	81.66 ± 4.52[a]	70.14 ± 3.85[a]	34.52 ± 1.75[a]
Group III	10.50 ± 0.86[a,b]	18.50 ± 0.83[a,b]	24.35 ± 0.89[a,b]	34.98 ± 2.26[a,b]	130.41 ± 9.62	125.62 ± 5.37[a,b]	96.84 ± 2.75[a,b]	80.42 ± 3.98[a,b]
Group IV	0.22 ± 0.03	0.20 ± 0.01	0.16 ± 0.03	0.16 ± 0.02	138.11 ± 10.60	130.58 ± 9.40	150.43 ± 6.45[b]	154.25 ± 12.64[b]

Values are expressed as the mean ± SEM, $n = 6$. [a]: Significant difference from Group I (control), $P \leq 0.01$; [b]: Significant difference from Group II, $P \leq 0.01$.

3.2. Reproductive organ weights

A significant decrease in the weight of the testis, epididymis, prostate gland and seminal vesicle was observed compared to the control group at all four time intervals (Table 2). The weight

Table 2
Reproductive organ weights (g) relative to body weight of rats treated with zinc and cadmium.

Organ	Time in week	Treatment			
		Control	Cd	Zn + Cd	Zn
Testis	2	0.930 ± 0.030	0.720 ± 0.020[a]	0.950 ± 0.040[b]	1.010 ± 0.120
	4	0.950 ± 0.010	0.590 ± 0.020[a]	0.900 ± 0.050[b]	0.980 ± 0.050
	6	0.960 ± 0.030	0.380 ± 0.010[a]	0.780 ± 0.040[a,b]	0.940 ± 0.080
	8	1.000 ± 0.050	0.290 ± 0.008[a]	0.810 ± 0.030[a,b]	0.980 ± 0.090
Vas deferens	2	0.070 ± 0.004	0.070 ± 0.003	0.070 ± 0.006	0.070 ± 0.001
	4	0.070 ± 0.002	0.060 ± 0.002	0.070 ± 0.003	0.060 ± 0.003
	6	0.080 ± 0.006	0.040 ± 0.001[a]	0.070 ± 0.002[b]	0.070 ± 0.004
	8	0.070 ± 0.001	0.030 ± 0.001[a]	0.050 ± 0.001[a,b]	0.080 ± 0.006
Epididymis	2	0.250 ± 0.008	0.150 ± 0.007[a]	0.240 ± 0.010	0.240 ± 0.010
	4	0.260 ± 0.009	0.110 ± 0.003[a]	0.180 ± 0.005[a,b]	0.270 ± 0.020
	6	0.240 ± 0.010	0.090 ± 0.003[a]	0.200 ± 0.010[a,b]	0.260 ± 0.010
	8	0.270 ± 0.010	0.080 ± 0.004[a]	0.170 ± 0.003[a,b]	0.320 ± 0.010[b]
Prostate	2	0.340 ± 0.010	0.160 ± 0.009[a]	0.250 ± 0.010[a,b]	0.310 ± 0.020
	4	0.350 ± 0.010	0.130 ± 0.004[a]	0.270 ± 0.010[a,b]	0.330 ± 0.010
	6	0.330 ± 0.010	0.080 ± 0.005[a]	0.240 ± 0.009[a,b]	0.340 ± 0.020
	8	0.360 ± 0.020	0.070 ± 0.004[a]	0.200 ± 0.004[a,b]	0.380 ± 0.030
Seminal vesicle	2	0.550 ± 0.020	0.300 ± 0.020[a]	0.420 ± 0.020[a,b]	0.570 ± 0.040
	4	0.560 ± 0.010	0.250 ± 0.010[a]	0.380 ± 0.010[a,b]	0.540 ± 0.050
	6	0.580 ± 0.040	0.160 ± 0.010[a]	0.260 ± 0.020[a,b]	0.590 ± 0.010
	8	0.570 ± 0.030	0.100 ± 0.007[a]	0.220 ± 0.010[a,b]	0.550 ± 0.030

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

of the vas deferens in the cadmium group decreased significantly after 6 and 8 weeks. The decrease in the weights of the reproductive organs was less pronounced in the rats given zinc and cadmium.

3.3. Sperm characteristics

A time-dependent decrease in sperm motility and count was noticed in the cadmium group in comparison to the control (Tables 3 and 4). Azoospermia was recorded in rats of the cadmium group at 8 weeks. The sperm abnormalities presented are shown in Tables 3 and 4. The cadmium group rats exhibited a significant elevation in sperm abnormalities (head, tail, total), whereas zinc treatment appeared to alleviate the deleterious effects of cadmium on sperm.

3.4. Hormone levels

The treatment of rats with zinc alleviated the alterations in the sex hormone levels induced by cadmium (Tables 5–7). Our results indicated a significant reduction in the testosterone level in Group II, by 40%, 42%, 69% and 94% after 2, 4, 6 and 8 weeks, respectively, compared to the control. The testosterone levels of Group III were still significantly decreased compared to the control (by 15%, 34% and 72% after 4, 6 and 8 weeks,

Table 5
Plasma FSH level (mIU/mL) of rats treated with zinc and cadmium.

Group	Plasma FSH level			
	Week 2	Week 4	Week 6	Week 8
Group I	2.53 ± 0.14	2.16 ± 0.19	2.72 ± 0.17	2.75 ± 0.10
Group II	3.06 ± 0.13[a]	4.49 ± 0.57[a]	4.97 ± 0.43[a]	6.54 ± 0.35[a]
Group III	2.71 ± 0.13[b]	2.90 ± 0.09[b]	3.13 ± 0.12[b]	2.99 ± 0.10[b]
Group IV	2.44 ± 0.12	2.45 ± 0.32	2.56 ± 0.45	2.90 ± 0.22

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

Table 6
Plasma LH level (mIU/mL) of rats treated with zinc and cadmium.

Group	Plasma LH level			
	Week 2	Week 4	Week 6	Week 8
Group I	2.99 ± 0.08	2.91 ± 0.10	2.81 ± 0.09	2.68 ± 0.11
Group II	2.44 ± 0.10[a]	2.00 ± 0.04[a]	1.61 ± 0.06[a]	1.26 ± 0.04[a]
Group III	2.95 ± 0.06[b]	2.42 ± 0.04[a,b]	2.12 ± 0.03[a,b]	1.96 ± 0.04[a,b]
Group IV	2.68 ± 0.15	2.75 ± 0.17	2.78 ± 0.13	2.79 ± 0.34

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

Table 3
Effect of zinc on sperm motility and sperm count of rats treated with cadmium.

Group	Sperm motility (%)				Sperm count per epididymis ($\times 10^6$)			
	Week 2	Week 4	Week 6	Week 8	Week 2	Week 4	Week 6	Week 8
Group I	85.14 ± 1.04	86.22 ± 0.94	84.86 ± 1.12	83.14 ± 1.32	25.33 ± 0.82	24.88 ± 0.78	27.96 ± 0.59	26.91 ± 0.64
Group II	74.78 ± 2.54[a]	40.83 ± 2.05[a]	21.15 ± 1.30[a]	–	22.39 ± 0.57	14.21 ± 0.67[a]	6.12 ± 0.33[a,b]	–
Group III	85.36 ± 1.73[b]	73.96 ± 2.72[a,b]	65.30 ± 3.01[a,b]	58.21 ± 2.36[a,b]	23.21 ± 0.80	19.04 ± 0.64[a,b]	12.26 ± 0.85[a,b]	9.88 ± 0.27[a,b]
Group IV	87.11 ± 6.23	88.06 ± 4.50	85.34 ± 2.43	83.45 ± 7.12	24.45 ± 2.32	25.22 ± 1.56	28.65 ± 1.13	31.23 ± 0.89[b]

Values are expressed as mean ± SEM, $n = 6$. –: Azoospermia; [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

Table 4
Sperm abnormalities (%) of rats treated with zinc and cadmium.

Group	Sperm	Sperm abnormalities			
		Week 2	Week 4	Week 6	Week 8
Group I	Head	2.59 ± 0.15	2.11 ± 0.16	1.96 ± 0.11	2.00 ± 0.07
	Tail	1.77 ± 0.09	1.59 ± 0.10	1.60 ± 0.09	1.70 ± 0.11
	Total	4.27 ± 0.22	3.16 ± 0.19	3.56 ± 0.20	3.70 ± 0.35
Group II	Head	16.05 ± 0.39[a]	20.74 ± 1.00[a]	31.00 ± 1.24[a]	–
	Tail	2.69 ± 0.16[a]	4.32 ± 0.28[a]	7.05 ± 0.40[a]	–
	Total	18.66 ± 0.38[a]	24.99 ± 1.01[a]	38.74 ± 1.76[a]	–
Group III	Head	3.20 ± 0.34[a,b]	7.89 ± 0.23[a,b]	8.98 ± 0.66[a,b]	12.76 ± 0.89[a,b]
	Tail	2.00 ± 0.10[a]	1.98 ± 0.12[a]	2.75 ± 0.18[a,b]	3.41 ± 0.22[a,b]
	Total	5.20 ± 0.34[a]	9.73 ± 0.25[a,b]	11.17 ± 0.68[a,b]	16.07 ± 1.00[a,b]
Group IV	Head	2.60 ± 0.14	2.17 ± 0.23	1.20 ± 0.02[a]	1.42 ± 0.05[a]
	Tail	1.66 ± 0.18	1.51 ± 0.13	1.25 ± 0.08[a]	1.50 ± 0.10[a]
	Total	4.47 ± 0.30	3.65 ± 0.22	2.40 ± 0.07[a]	2.86 ± 0.10[a]

Values are expressed as mean ± SEM, $n = 6$. –: Azoospermia; [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

Table 7
Plasma testosterone level (ng/mL) of rats treated with zinc and cadmium.

Group	Plasma testosterone level			
	Week 2	Week 4	Week 6	Week 8
Group I	2.99 ± 0.08	2.91 ± 0.10	2.81 ± 0.09	2.68 ± 0.11
Group II	2.44 ± 0.10[a]	2.00 ± 0.04[a]	1.61 ± 0.06[a]	1.26 ± 0.04[a]
Group III	2.95 ± 0.06[b]	2.42 ± 0.04[a,b]	2.12 ± 0.03[a,b]	1.96 ± 0.04[a,b]
Group IV	2.68 ± 0.15	2.75 ± 0.17	2.78 ± 0.13	2.79 ± 0.34

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

respectively), but did not change significantly after 2 weeks. The values for testosterone levels in Group III, however, were significantly higher than those of Group II.

3.5. Oxidative stress markers

The effect of zinc on oxidative stress parameters in rats given cadmium is presented in Tables 8–10. Zinc significantly reduced blood hydroperoxide, testicular MDA (markers of LPO), TNF-α and NO in rats treated with cadmium. Furthermore, levels of testicular CAT, SOD, NO and ascorbic acid were significantly elevated in Group III compared to Group II.

Although LH levels lowered significantly in Group III compared to the control, they were significantly higher than those in the cadmium group. Subcutaneous injection of rats with cadmium, meanwhile, led to a significant increase in FSH level compared to the control and these increased levels did not change significantly in rats treated with zinc and cadmium in combination.

3.6. Histological studies

Histological investigation of the testes of the control group rats showed apparently normal seminiferous tubules, spermatogenic cells and interstitial cells with Leydig cells (Figure 1A). The rats treated with cadmium, however, showed many histopathological changes, including degeneration and desquamation of the spermatogenic cells lining the seminiferous tubules and a dilation of the interstitial spaces with degeneration of interstitial cells. In addition, cadmium induced a reduction of spermatozoa produced in the lumen of the seminiferous tubules. Pyknotic nuclei of seminiferous epithelium were also observed (Figure 1B).

Table 8
Blood hydroperoxide level (mg/100 mL) of rats treated with zinc and cadmium.

Group	Blood hydroperoxide level			
	Week 2	Week 4	Week 6	Week 8
Group I	25.84 ± 1.32	26.53 ± 1.06	26.00 ± 0.98	24.95 ± 0.88
Group II	38.79 ± 1.06[a]	45.02 ± 2.72[a]	47.44 ± 1.68[a]	55.80 ± 0.71[a]
Group III	32.19 ± 1.73[a,b]	33.84 ± 1.35[a,b]	37.52 ± 1.10[a,b]	38.40 ± 0.80[a,b]
Group IV	28.43 ± 1.87	22.30 ± 2.02	22.60 ± 1.54	26.11 ± 2.73

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

Table 9
Testicular MDA (nmoL/g tissue) of rats treated with zinc and cadmium.

Group	Testicular MDA			
	Week 2	Week 4	Week 6	Week 8
Group I	18.43 ± 0.81	17.43 ± 0.53	19.11 ± 0.41	20.56 ± 0.36
Group II	21.65 ± 0.09[a]	28.14 ± 0.17[a]	31.65 ± 0.78[a]	38.75 ± 0.62[a]
Group III	19.05 ± 1.11	20.55 ± 0.65[a,b]	22.36 ± 0.36[a,b]	25.24 ± 0.37[a,b]
Group IV	20.05 ± 2.50	19.46 ± 1.15	20.33 ± 1.65	16.28 ± 0.48[a]

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

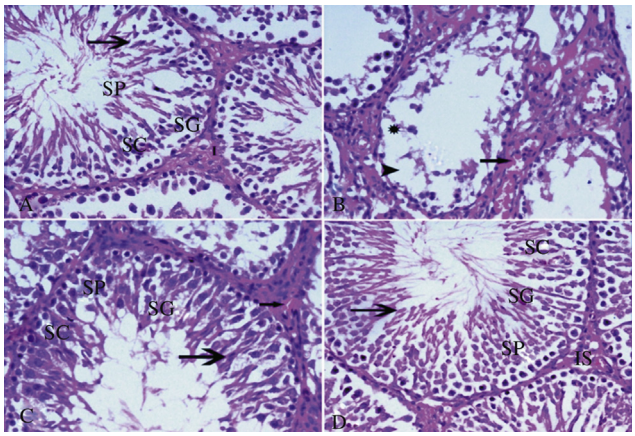

Figure 1. Testicular histopathology of rats treated with zinc and cadmium. A: Photomicrograph of the testis of rat from control group is showing normal seminiferous tubule with different stages of spermatogenic cells; B: Photomicrograph of the testis of rat from group treated with cadmium showing degeneration of spermatogenic cells lining seminiferous tubules with desquamation of spermatogenic cells, interstitial spaces were widening with degeneration of Leydig cells; Vacuolization of the seminiferous epithelium (arrowhead) and pyknotic nuclei (star) were also observed; C: Photomicrograph of the testis of rat from group treated with cadmium and zinc showing partially restoration of spermatogenic cells. Few pyknotic nuclei of some spermatogenic cells and the interstitial congestion were still found (short arrow); D: Photomicrograph of the testis of rat from group treated with zinc (H & E, 400×) showed normal structure; SG: Spermatogonia; SP: Spermatid elongated spermatid (arrow); SC: Sertoli cells; IS: Interstitial space appear normal.

Table 10
Testicular oxidative stress parameters of rats treated with zinc and cadmium.

Group	TNF-α (pg/mg protein)	NO (nmoL/100 mg tissue)	Ascorbic acid (μg/g tissue)	GSH (μg/mg protein)	SOD (Unit/mg protein)	CAT (μmol/min/mg protein)
Group I	3.41 ± 0.10	85.35 ± 2.86	200.67 ± 13.08	5.80 ± 0.12	21.74 ± 0.85	8.10 ± 0.37
Group II	14.24 ± 1.03[a]	176.20 ± 5.91[a]	105.87 ± 6.20[a]	1.56 ± 0.03[a]	7.34 ± 0.29[a]	3.76 ± 0.17[a]
Group III	6.23 ± 0.21[ab]	121.05 ± 3.67[ab]	174.89 ± 5.94[ab]	3.50 ± 0.06[ab]	14.31 ± 0.42[ab]	5.63 ± 0.17[ab]
Group IV	3.17 ± 0.25	92.43 ± 6.15	220.54 ± 7.39	5.34 ± 0.54	26.87 ± 1.06[a]	8.50 ± 2.98

Values are expressed as mean ± SEM, $n = 6$. [a]: Significant difference from control, $P \leq 0.01$; [b]: Significant difference from cadmium group, $P \leq 0.01$.

After treatment with zinc, these histopathological changes in the spermatogenic cells were partially reversed, although a few pyknotic nuclei and some interstitial congestion were still found (Figure 1C). Rats treated with zinc and cadmium, however, showed more complete renovation of spermatogenic cells in most seminiferous tubules, and interstitial spaces occupied by Leydig cells with only mild congestion (Figure 1D).

3.7. Immunohistochemical studies

Photomicrograph of the testis of rat from control group showed no iNOS immunoreactivity (Figure 2A), while photomicrograph of the testis of rat from group treated with cadmium showed increased iNOS immunoreactivity (Figure 2B). Surprisingly, photomicrograph of the testis of rat from group treated with cadmium and zinc showed mild decreased iNOS immunoreactivity (Figure 2C), besides the testis of rat from group treated with zinc showed a decreased iNOS immunoreactivity (Figure 2D).

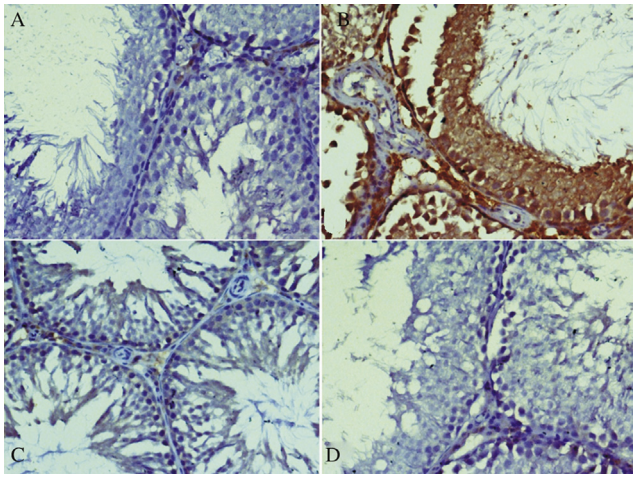

Figure 2. Testicular iNOS immunohistochemistry of rats treated with zinc and cadmium.
A: Photomicrograph of the testis of rat from Group I showing no iNOS immunoreactivity; B: Photomicrograph of the testis of rat from Group II indicating increased iNOS immunoreactivity; C: Photomicrograph of the testis of rat from Group III showing mild decreased iNOS immunoreactivity; D: Photomicrograph of the testis of rat from Group IV showing a decreased iNOS immunoreactivity (iNOS immunohistochemistry counterstained with hematoxylin, 400×).

4. Discussion

Since zinc is a micronutrient trace element acting as cellular antioxidant and a co-factor for many enzymes [4], it could provide an effective therapy to reduce testicular oxidative stress caused by cadmium. Our study aimed to evaluate the efficacy of zinc in reducing the toxicity of cadmium affected testicular antioxidant enzymes and inflammatory markers. In the present work, zinc was shown to reduce oxidative stress due to cadmium treatment as manifested by a decrease in blood hydroperoxide and testicular MAD (markers of LPO), and to improve the concentration of testicular GSH, CAT and SOD. The adjustment of LPO following zinc treatment is very probably due to its antioxidant properties [28]. LPO is considered to be a good indicator of oxidative stress [29].

Zinc has an intensive influence in the organization of cellular glutathione, which is vital to cellular antioxidant defense [30]. The antioxidant enzymes SOD and CAT provide a defense against oxidative cell injury. SOD is a metal-containing enzyme that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide. Therefore, it plays an important role in the defense against oxidative stress through quenching free radicals [31]. CAT, an enzyme found mainly in peroxisomes, degrades hydrogen peroxide to water and oxygen, thereby completing the reaction started by SOD.

Zinc possesses different antioxidant mechanisms. It protects oxidation of sulfhydryl groups and keep intracellular levels of GSH [32]; it also lowers the activities of oxidant promoting enzymes such as iNOS; it increases the activation of antioxidant enzymes such as CAT and SOD and also inhibits the generation of LPO products [33]. In addition, zinc induces synthesis of metallothionein, which is a powerful scavenger of free radicals [34], and it protects cell membrane against oxidative injuries [35].

In the present experiment, zinc treatment afforded additional protection by reducing the accumulation of testicular cadmium. A probably mechanism for this is through zinc's inducement of metallothionein synthesis, which can form a complex with cadmium, allowing it to be excreted by the kidney [36]. Zinc and cadmium also compete for a common transport mechanism in organisms, which can result in an increase in the excretion of cadmium [37].

Zinc reduced toxic effects of cadmium by decreasing cadmium absorption from the digestive tract [38]. Moreover, zinc maintains testicular zinc levels at an appropriate concentration and prevents the decrease in testicular ascorbic acid resulting from cadmium treatment. Both zinc and vitamin C are known to possess antioxidant activity [4,39] and a protective effect against cadmium toxicity [40,41], and it has also been reported that zinc deficiency increases cadmium-mediated testicular free radical damage [42].

Cadmium is a toxic metal, targeting the testes following acute intoxication and serving to alter the testicular antioxidant defense system leading to increase oxidative stress [43]. The decrease in testicular GSH, SOD, CAT, zinc and ascorbic acid shown in the present work may result in an increase in testicular LPO in cadmium intoxicated rats, since these antioxidants prevent peroxidation by removing reactive oxygen species. The histopathological changes observed in cadmium treated rats may result from increased oxidative stress [44].

The present study showed marked histopathological changes in the testicular tissue. However, many studies focusing on cadmium related changes in testicular histopathology have implicated testicular blood vessel damage, followed by the degeneration of spermatopoietic epithelial, as the main cause of cadmium toxicity [20,45].

The accumulated degenerated germ cells in the lumina of seminiferous tubules of cadmium group can be referred to failure of Sertoli cells to perform their function in engulfing these bodies [44–46]. The defects of Sertoli cells result in loss of spermatic cells and may lead to the destruction of testicular tissue and infertility [47]. Moreover, Al-Ani *et al.* [48] reported that co-treatment with zinc ameliorated the toxic effect of cadmium on spermatogenesis.

Zinc affords protection against cadmium toxicity by keeping normal redox balance inside the cell [49] and DNA maintenance [32]. Cadmium can also alter sex hormone levels by affecting the pituitary–testicular axis. We observed a decrease in plasma testosterone and LH levels in the cadmium group, which was associated with an increase in FSH level. Sen Gupta *et al.* [50] concluded that the decline in the testosterone level in cadmium treated rats can result from the decrease in the activity of steroidogenic enzymes. The increase of FSH level can be attributed to feedback mechanism of low level of testosterone on anterior pituitary gland. It has been found that testosterone has direct negative feedback effects on the anterior pituitary gland. This is achieved through making the anterior pituitary less responsive to gonadotropin-releasing hormone [51]. Higher concentration of FSH in the infertile men is associated with azoospermia [52].

The significant decrease in the weights of the testis and associated sex organs in rats treated with cadmium only can be attributed to a decrease in the testosterone level, since these organs are androgen dependent organs [53]. The present experiment demonstrated that hormone levels and the weight of the sex organs improved in rats treated with zinc and cadmium, indicating that zinc protects the reproductive system against the toxic effects of cadmium. The present experiment also showed a decrease in sperm motility and sperm count in rats treated with cadmium, along with an increase in sperm abnormalities.

As plasma membrane of spermatozoa contains large amounts of polyunsaturated fatty acid, it is liable to the reactive oxygen species arising from cadmium treatment [54]. Increased sperm membrane LPO has been shown to decrease sperm motility and elevate sperm abnormalities [55]. The decrease in the sperm count in the cadmium group could be related to the low level of testosterone, which is a regulator for sperm production [56]. Zinc's alleviation of the toxic effects of cadmium on sperm is probably due to its antioxidant properties or its ability to improve sex hormone levels in cadmium intoxicated rats.

The production of different inflammatory mediators is increased by inflammatory stimuli, where they play a vital role in regulating Leydig cell function and spermatogenesis [57,58]. Some researchers have shown that the NO is capable of inhibiting steroidogenesis by Leydig cells and adrenal cortex [59,60]. Production of NO occurs through the action of the NOS enzyme. An immunohistochemical study has shown that NOS is present in rat testes [61]. Our study indicates an increase in testicular iNOS expression in the group treated with cadmium, resulting in an increase in testicular NO concentrations. Similarly, testicular TNF-α levels increased in the cadmium group.

TNF-α can induce production of reactive oxygen species which led to decreased sperm motility [62,63]. Excess NO reacts with superoxide anions to generate peroxynitrite radicals that cause further cell damage. Also, excess NO lowered intracellular GSH which leads to an increased susceptibility to oxidative stress [64]. The observed improvement in sperm parameters, hormone levels and histology of the testis in the zinc and cadmium group can be attributed to a reduction in the elevation of NO and TNF-α caused by zinc, together with its role in the preservation of testicular antioxidant enzymes, GSH, zinc and ascorbic acid.

In conclusion, zinc served to ameliorate cadmium induced testicular toxicity by reducing LPO (MDA and hydroperoxide) and inflammatory mediators (NO, iNOS, TNF-α) and by helping to maintain the status of the testicular antioxidant defense system.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This project was supported by King Saud University, Deanship of Scientific Research, College of Science Research Centre.

References

[1] MacDonald RS. The role of zinc in growth and cell proliferation. *J Nutr* 2000; **130**(5S Suppl): 1500S–8S.
[2] Prasad AS. Zinc in human health: effect of zinc on immune cells. *Mol Med* 2008; **14**(5–6): 353-7.
[3] Zheng J, Zhang Y, Xu W, Luo Y, Hao J, Shen XL, et al. Zinc protects HepG2 cells against the oxidative damage and DNA damage induced by ochratoxin A. *Toxicol Appl Pharmacol* 2013; **268**(2): 123-31.
[4] Prasad AS. Zinc is an antioxidant and anti-inflammatory agent: its role in human health. *Front Nutr* 2014; **1**: 14.
[5] Cruz KJ, de Oliveira AR, Marreiro Ddo N. Antioxidant role of zinc in diabetes mellitus. *World J Diabetes* 2015; **6**(2): 333-7.
[6] Sato M, Bremner I. Oxygen free radicals and metallothionein. *Free Radic Biol Med* 1993; **14**(3): 325-37.
[7] Bogani D, Morgan MA, Nelson AC, Costello I, McGouran JF, Kessler BM, et al. The PR/SET domain zinc finger protein Prdm4 regulates gene expression in embryonic stem cells but plays a nonessential role in the developing mouse embryo. *Mol Cell Biol* 2013; **33**(19): 3936-50.
[8] Alam S, Kelleher SL. Cellular mechanisms of zinc dysregulation: a perspective on zinc homeostasis as an etiological factor in the development and progression of breast cancer. *Nutrients* 2012; **4**(8): 875-903.
[9] Sidhu P, Garg ML, Dhawan DK. Time dependent study to evaluate the efficacy of zinc on hepatic marker enzymes and elemental profile in serum and liver of protein deficient rats. *Biometals* 2005; **18**(1): 97-106.
[10] Ozdemir G, Inanc F. Zinc may protect remote ocular injury caused by intestinal ischemia reperfusion in rats. *Tohoku J Exp Med* 2005; **206**(3): 247-51.
[11] Caglar OM, Bilgihan A, Turkcu UO, Biberoglu G, Take G. The effect of zinc on ethanol-induced oxidative stress in rat liver. *Turk J Biochem* 2012; **37**(4): 437-44.
[12] Cheah Y, Yang W. Functions of essential nutrition for high quality spermatogenesis. *Adv Biosci Biotechnol* 2011; **2**(4): 182-97.
[13] Omu AE, Al-Azemi MK, Al-Maghrebi M, Mathew CT, Omu FE, Kehinde EO, et al. Molecular basis for the effects of zinc deficiency on spermatogenesis: an experimental study in the Sprague-Dawley rat model. *Indian J Urol* 2015; **31**(1): 57-64.
[14] Kamunde C, MacPhail R. Metal-metal interactions of dietary cadmium, copper and zinc in rainbow trout, *Oncorhynchus mykiss*. *Ecotoxicol Environ Saf* 2011; **74**(4): 658-67.
[15] Jeon YW, Choi BS, Park JD. The effects of dietary zinc deficiency on the expression of metallothionein, absorption and distribution of cadmium in rats. *J Environ Toxicol* 2004; **19**(2): 191-200.
[16] Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. *Toxicology* 2011; **283**(2–3): 65-87.
[17] Strumylaite L, Mechonosina K. Cadmium carcinogenesis – some key points. *Environ Med* 2011; **14**(3): 13-5.

[18] Lacorte LM, Seiva FR, Rinaldi JC, Delella FK, Moroz A, Sarobo C, et al. Caffeine reduces cadmium accumulation in the organism and enhances the levels of antioxidant protein expression in the epididymis. *Reprod Toxicol* 2013; **35**: 137-43.
[19] Goyer RA, Liu J, Waalkes MP. Cadmium and cancer of prostate and testis. *Biometals* 2004; **17**(5): 555-8.
[20] Adamkovicova M, Toman R, Cabaj M, Massanyi P, Martiniakova M, Omelka R, et al. Effects of subchronic exposure to cadmium and diazinon on testis and epididymis in rats. *ScientificWorldJournal* 2014; **2014**: 632581.
[21] Kaur S, Sharma S. Evaluation of toxic effect of cadmium on sperm count, sperm motility and sperm abnormality in albino mice. *Int J Adv Res* 2015; **3**(3): 335-43.
[22] Bonda E, Wlostowski T, Krasowska A. Testicular toxicity induced by dietary cadmium is associated with decreased testicular zinc and increased hepatic and renal metallothionein and zinc in the bank vole (*Clethrionomys glareolus*). *Biometals* 2004; **17**(6): 615-24.
[23] Abdel-Hady EK, Abdel-Rahman GH. Protective effect of coenzyme Q10 on cadmium-induced testicular damage in male rabbits. *Am Eur J Toxicol Sci* 2011; **3**(3): 153-60.
[24] Onwuka FC, Erhabor O, Eteng MU, Umoh IB. Ameliorative effect of cabbage extract on cadmium-induced changes on hematology and biochemical parameters of albino rats. *J Toxicol Environ Health Sci* 2010; **2**(2): 11-6.
[25] Ebaid H, Hassan I, Bashandy S, Taha NA, Mahmood A, Alomar S, et al. Zinc improves the immune function and the proliferation of lymphocytes in cadmium-treated rats. *Cent Eur J Immunol* 2014; **39**(4): 441-8.
[26] Narayana K, Prashanthi N, Nayanatara A, Kumar HH, Abhilash K, Bairy KL. Effects of methyl parathion (o,o-dimethyl o-4-nitrophenyl phosphorothioate) on rat sperm morphology and sperm count, but not fertility, are associated with decreased ascorbic acid level in the testis. *Mutat Res* 2005; **588**(1): 28-34.
[27] Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. *Methods Enzymol* 1979; **62**: 3-11.
[28] Pathak A, Rathore AS, Bhutani V, Pathak R. Role of zinc on antioxidative enzymes and lipid peroxidation in brain of diabetic rats. *J Drug Metab Toxicol* 2012; http://dx.doi.org/10.4172/2157-7609.1000122.
[29] Saygili EI, Konukoglu D, Papila C, Akcay T. Levels of plasma vitamin E, vitamin C, TBARS, and cholesterol in male patients with colorectal tumors. *Biochemistry (Mosc)* 2003; **68**(3): 325-8.
[30] Duprez J, Roma LP, Close AF, Jonas JC. Protective antioxidant and antiapoptotic effects of $ZnCl_2$ in rat pancreatic islets cultured in low and high glucose concentrations. *PLoS One* 2012; **7**(10): e46831.
[31] Fridovich I. Superoxide anion radical ($O_2^{-\cdot}$), superoxide dismutases, and related matters. *J Biol Chem* 1997; **272**(30): 18515-7.
[32] Celino FT, Yamaguchi S, Miura C, Ohta T, Tozawa Y, Iwai T, et al. Tolerance of spermatogonia to oxidative stress is due to high levels of Zn and Cu/Zn superoxide dismutase. *PLoS One* 2011; **6**(2): e16938.
[33] Bao B, Ahmad A, Azmi A, Li Y, Prasad A, Sarkar FH. The biological significance of zinc in inflammation and aging. In: Rahman I, Bagchi D, editors. *Inflammation, advancing age and nutrition*. San Diego: Academic Press; 2014, p. 15-27.
[34] Suntres ZE, Lui EM. Biochemical mechanism of metallothionein – carbon tetrachloride interaction *in vitro*. *Biochem Pharmacol* 1990; **39**(5): 833-40.
[35] Bettger WJ, O'Dell BL. A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci* 1981; **28**(13): 1425-38.
[36] Kägi JH. Overview of metallothionein. *Methods Enzymol* 1991; **205**: 613-26.
[37] Mohamed D, Saber A, Omar A, Soliman A. Effect of cadmium on the testes of adult albino rats and the ameliorating effect of zinc and vitamin E. *Br J Sci* 2014; **11**(1): 72-95.
[38] Babaknejad N, Moshtaghie AA, Shahanipour K, Somaye B. The protective roles of zinc and magnesium in cadmium-induced renal toxicity in male Wistar rats. *Iran J Toxicol* 2015; **8**(27): 1160-7.
[39] Layachi N, Kechrid Z. Combined protective effect of vitamins C and E on cadmium induced oxidative liver injury in rats. *Afr J Biotechnol* 2012; **11**(93): 16013-20.
[40] Bashandy SA, Alhazza IM, Mubark M. Role of zinc in the protection against cadmium induced hepatotoxicity. *Int J Pharmacol* 2006; **2**(1): 79-88.
[41] El-Refaiy AI, Eissa FI. Protective effects of ascorbic acid and zinc against cadmium-induced histopathological, histochemical and cytogenetic changes in rats. *Comun Sci* 2012; **3**(3): 162-80.
[42] Oteiza PI, Adonaylo VN, Keen CL. Cadmium-induced testes oxidative damage in rats can be influenced by dietary zinc intake. *Toxicology* 1999; **137**(1): 13-22.
[43] Rajendar B, Bharavi K, Rao GS, Kishore PV, Kumar PR, Kumar CS, et al. Protective effect of alpha-tocopherol on biochemical and histological alterations induced by cadmium in rat testes. *Indian J Physiol Pharmacol* 2011; **55**(3): 213-20.
[44] EL-Shahat AE, Gabr A, Meki AR, Mehana ES. Altered testicular morphology and oxidative stress induced by cadmium in experimental rats and protective effect of simultaneous green tea extract. *Int J Morph* 2009; **27**(3): 757-64.
[45] Messaoudi I, Banni M, Saïd L, Saïd K, Kerkeni A. Evaluation of involvement of testicular metallothionein gene expression in the protective effect of zinc against cadmium-induced testicular pathophysiology in rat. *Reprod Toxicol* 2010; **29**(3): 339-45.
[46] Kamel MM, Abd El Razek AH, Ahmed KA, Kamel GM. Exposure of adult male rats to cadmium: assessment of sexual behaviour, fertility, aggression as well as anxiety like behaviour with special reference to biochemical and pathological alterations. *Life Sci J* 2011; **8**(2): 106-19.
[47] Farombi EO, Adedara IA, Akinrinde SA, Ojo OO, Eboh AS. Protective effects of kolaviron and quercetin on cadmium-induced testicular damage and endocrine pathology in rats. *Andrologia* 2012; **44**(4): 273-84.
[48] Al-Ani NK, Al-Kawaz U, Saeed BT. Protective influence of zinc on reproductive parameters in male rat treated with cadmium. *Am J Med Sci* 2015; **5**(2): 73-81.
[49] Souza V, Escobar Mdel C, Bucio L, Hernandez E, Gutiérrez-Ruiz MC. Zinc pretreatment prevents hepatic stellate cells from cadmium-produced oxidative damage. *Cell Biol Toxicol* 2004; **20**(4): 241-51.
[50] Sen Gupta R, Kim J, Gomes C, Oh S, Park J, Im WB, et al. Effect of ascorbic acid supplementation on testicular steroidogenesis and germ cell death in cadmium-treated male rats. *Mol Cell Endocrinol* 2004; **221**(1–2): 57-66.
[51] Votenko AM. [Research development of problem "Scientific principles of hygiene and physiology of human adaptation to conditions of the World's ocean"]. *Gig Sanit* 1993; **2**: 8-10. Russian.
[52] Eniola OW, Adetola AA, Olufemi AA, Oladipupo MA. Evaluation of hormonal and physical factors responsible for male infertility in Sagamu South Western Nigeria. *Der Pharm Lett* 2012; **4**(5): 1475-9.
[53] Trentacoste SV, Friedman AS, Youker RT, Breckenridge CB, Zirkin BR. Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats. *J Androl* 2001; **22**(1): 142-8.
[54] Alvarez JG, Storey BT. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev* 1995; **42**(3): 334-46.
[55] Kashmiri ZN. Reactive oxygen species and fertility in male. *World J Pharm Pharm Sci* 2015; **4**(9): 379-87.
[56] Steinberger E. Hormonal regulation of the seminiferous tubule function. *Curr Top Mol Endocrinol* 1975; **2**: 337-52.
[57] Zhang H, Yin Y, Wang G, Liu Z, Liu L, Sun F. Interleukin-6 disrupts blood-testis barrier through inhibiting protein degradation or activating phosphorylated ERK in Sertoli cells. *Sci Rep* 2014; **4**: 4260.
[58] Hales DB. Leydig cell-macrophage interactions: an overview. In: Payne AH, Hardy MP, Russell LD, editors. *The Leydig cell*. Vienna: Cache River Press; 1996, p. 451-65.
[59] Sokanovic SJ, Baburski AZ, Janjic MM, Stojkov NJ, Bjelic MM, Lalosevic D, et al. The opposing roles of nitric oxide and cGMP in the age-associated decline in rat testicular steroidogenesis. *Endocrinology* 2013; **154**(10): 3914-24.

[60] Cameron LA, Hinson JP. The role of nitric oxide derived from L-arginine in the control of steroidogenesis, and perfusion medium flow rate in the isolated perfused rat adrenal gland. *J Endocrinol* 1993; **139**(3): 415-23.

[61] Liman N, Alan E, Beyaz F, Gurbulak K. Endothelial and inducible nitric oxide synthase (NOS) immunoreactivity and NOS-associated NADPH-diaphorase histochemistry in the domestic cat (*Felis catus*) testis. *Theriogenology* 2013; **80**(9): 1017-32.

[62] Koçak I, Yenisey C, Dundar M, Okyay P, Serter M. Relationship between seminal plasma interleukin-6 and tumor necrosis factor alpha levels with semen parameters in fertile and infertile men. *Urol Res* 2002; **30**(4): 263-7.

[63] Fraczek M, Sanocka D, Kamieniczna M, Kurpisz M. Proinflammatory cytokines as an intermediate factor enhancing lipid sperm membrane peroxidation in *in vitro* conditions. *J Androl* 2008; **29**(1): 85-92.

[64] Magierowski M, Magierowska K, Kwiecien S, Brzozowski T. Gaseous mediators nitric oxide and hydrogen sulfide in the mechanism of gastrointestinal integrity, protection and ulcer healing. *Molecules* 2015; **20**(5): 9099-123.