Effect of astaxanthin nanoparticles in protecting the post-thawing quality of rooster sperm challenged by cadmium administration

Davood Najafi,[*] Ramezan Ali Taheri,[*,1] Abouzar Najafi,[†] Mohammad Shamsollahi,[†] and Manuel Alvarez-Rodriguez[‡,§]

*Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, 17166 Tehran, Iran; [†]Department of Animal Science, College of Agriculture, University of Tabriz, 51666 Tabriz, Iran; [‡]Department of Clinical and Experimental Medicine (IKE), Division of Clinical Sciences, Obstetrics and Gynecology, Linköping University, 58113 Linköping, Sweden; and [§]Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

ABSTRACT The protective role of astaxanthin nanoparticles (**Ast NPs**, 25 mg/kg p.o) against cadmium (**Cd**, 1 mg/100 g b.w. SC), a known inductor of lipid peroxidation and changes in the antioxidant defense system in the Ross 308 breeder roosters sperm, was examined. Sperm motility (computer-assisted sperm motility analysis), membrane integrity (hypoosmotic swelling test), viability, total abnormality, and enzymatic parameters were assessed after thawing. The testis/body weight (mg/kg) ratio and HE staining results of testis were also performed. The obtained results showed that Cd induced detrimental effects on testis and sperm, while Cd treated by Ast NPs (Cd Ast) diminished this change compared to the Cd group. Cd-treated group resulted in significantly ($P < 0.05$) lowest total (37.29 ± 2.46) and progressive (5.84 ± 0.47) motility and decreased antioxidant enzyme activity (CAT, TAC, and GPx), as well as producing a significant ($P < 0.05$) decrease in testis weight (mg) compared to the control group. Treatment with Ast NPs (Ast NPs + Cd) had reversed Cd-induced changes in the antioxidant defense system and significantly prevented Cd-induced testis damage. In conclusion, the results of our work suggest that Ast NPs at 25 mg/kg act as a potent antioxidant in protecting rooster testes against oxidative stress induced by Cd.

Key words: astaxanthin, nanoparticle, cadmium, rooster, toxicity

2020 Poultry Science 99:1678–1686
https://doi.org/10.1016/j.psj.2019.12.006

INTRODUCTION

The nanotechnology industry has matured and expanded at a rapid pace in the last decade, leading to the research and development of nanomaterials with enormous potential (Bagdeli et al., 2017; Fasihi-Ramandi et al., 2018; Hajipour et al., 2018). Nanoparticles (**NPs**), due to their nanoscale and huge surface area, may interact with biological systems by more efficient approaches (Azin et al., 2017; Khansary et al., 2017). The improved drug bioavailability of NPs is attributed to the fact that particles in the nanosize range are efficient in crossing permeability barriers. It has been demonstrated that absorption and bioavailability of some drugs were significantly enhanced when administered as NPs (Dian et al., 2013; Sood et al., 2013; Rasaie et al., 2014). Liquid storage of avian semen is a technique utilized in poultry to optimize the management of genetically superior males (Rad et al., 2016). The cell membrane of avian sperm contains high amounts of polyunsaturated fatty acids that can easily undergo lipid peroxidation (**LPO**) in the presence of reactive oxygen species (**ROS**), thus making avian spermatozoa highly susceptible to LPO (Cerolini et al., 1997, 2006; Fattah et al., 2017). The effects of LPO include irreversible loss in motility, and damage to spermatozoa DNA and performance (Rad et al., 2016; Mehdipour et al., 2017; Najafi et al., 2019a). Antioxidant supplementation of semen extenders, for the purposes of LPO inhibition and maintenance of membrane integrity during semen storage, has previously been described in many mammalian and bird species (Partyka et al., 2013; Najafi et al., 2014; Swami et al., 2017).

Cadmium (**Cd**), one of the most toxic heavy metals, has no known beneficial biological function, and it poses a significant public health hazard, including

[1]Corresponding author: taheri@bmsu.ac.ir

Table 1. Detailed composition of the basic freezing medium extender.

Ingredient	
Potassium citrate tribasic monohydrate (g)	0.64
Sodium-L-glutamate (g)	8.67
Magnesium chloride anhydrous (g)	0.34
D-(−)-Fructose (g)	5
Potassium phosphate dibasic trihydrate (g)	7.59
Potassium phosphate monobasic (g)	0.7
N-[Tris (hydroxymethyl) methyl]-2	2.71
Sodium acetate trihydrate (g)	3.1
Soybean lecithin (%)	1
Glycerol (%)	8
Purified water (mL)	1,000
pH	7.1
Osmolarity (mOsm/kg)	310

reproductive toxicity (Benoff et al., 2008). Cd poisoning has been reported in individuals engaged in the battery and paint processing industries. Testicular injury is a major symptom of Cd poisoning, and it also results in blood–testes barrier destruction, germ cell apoptosis, testicular edema, hemorrhaging, necrosis, increased LPO, and infertility (Siu et al., 2009; de Souza Predes et al., 2010; Alkhedaide et al., 2016). The Cd reproductive-injury effect has been considered by numerous studies in recent decades in species including fish (Acosta et al., 2016), mouse, and rat (Sayed et al., 2014; Wang et al., 2017; Zhang et al., 2017), as well as in humans (Li et al., 2016; Riaz et al., 2016; Zhou et al., 2016), avian (Massanyi et al., 2008), and bovine (Tvrda et al., 2012, 2013), which most of them have focused on strategies to modify testicular damages. Using antioxidants such as sulforaphane (Yang et al., 2016), quercetin, and catechin (Jamalan et al., 2016), coenzyme Q10 and vitamin E (Ognjanović et al., 2010) are among those strategies to decrease Cd-induced testicular damage and improve sperm recovery post-cryopreservation. Moreover, it has been shown that various antioxidants protect cells from Cd-induced toxicity, by reducing GSH, SOD, and MDA presence (Tandon et al., 2003).

Recently, different types of nanotechnology-based methods, including nanoemulsion, have been used for delivering drugs to the targeted site without any adverse effects. The antioxidant activity of astaxanthin (**Ast**) has been shown to be 10 times that of lutein, canthaxanthin, and b-carotene. Astaxanthin like other carotenoids is very lipophilic compound which recorded low bioavailability. Therefore, a nanopreparation of Ast has been developed that is more stable and well bioavailable. However, the current study investigates the protective effects of Ast, as a lipid-soluble carotenoid against Cd-induced toxicity in rooster testis and reduced sperm quality. Recently, the red carotenoid pigment Ast, a potent antioxidant without provitamin-A activity present in some marine organisms (Lai et al., 2004), has been used in numerous studies to improve sperm quality (Mansour et al., 2006; Fang et al., 2015; Tizkar et al., 2015b; Vahidinia et al., 2017). Furthermore, it has been reported in the literature that the antioxidant activity of Ast is approximately 10 times higher than other carotenoids such as zeaxanthin, lutein, canthaxanthin, and β-carotene and about 100 times than that of α-tocopherol (Miki, 1991).

Thus, the main aim of the present study was to investigate the protective effect of Ast NPs in 1) roster sperm cryopreservation and 2) against LPO, ROS production, changes in the enzymatic (MDA, CAT, SOD, TAC, and GPX) antioxidant activity of fresh and post-thawed sperm, and testicular damage Cd-induced model in rooster.

MATERIALS AND METHODS

Chemicals

All materials and chemicals were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich Corporation (St Louis, MO).

Animals and Treatment

A total of 20 adult male Ross 308 breeder roosters (25 wk old) were selected from a commercial flock and were housed individually in cages (70 × 70 × 85 cm) at 18 to 22°C in a 15 L: 09 D photoperiod program. The animals were fed on a diet composed of 10% crude protein, 3,170 kcal ME/kg, 0.9% calcium, and 0.45% accessible phosphate without any additional antioxidant in feed. All birds were handled in accordance with methods approved by the Animal Care and Use Committee of Baqiyatallah University of Medical Sciences.

The roosters were divided into 4 experimental groups each consisting of 5 animals and were treated as follows: group 1– control roosters (treated subcutaneously (SC) with isotonic saline); group 2– Cd (received i.p. a single dose of 1 mg Cd/100 g body weight in 0.1 mL isotonic saline once a week) subcutaneously (SC); group 3—Ast NPs 25 mg/kg peroral (p.o) for 5 consecutive days a week for 5 wk; group 4—a single dose of Cd (1 mg/100 g b.w. SC) + Ast NPs (25 mg/kg p.o) for 5 consecutive days a week for 5 wk.

Roosters received, twice a day in the morning and the evening, approximately 90 g of diet within the experiment period. Water was provided ad libitum.

Semen Collection

Semen samples were collected weekly from the roosters, after 2-wk habituation, by the dorso-abdominal massage method (Burrows and Quinn, 1937). Briefly, ejaculates were collected using the dorso-abdominal massage method. The semen was caught in a 15-mL graduated centrifuge tubes, which is transferred immediately to the laboratory. To maintain the temperature of the ejaculates, the tubes was placed in a water bath (37°C), for primary evaluation in the laboratory. Normal quality of sperm was approved in the experiment if the subsequent parameters were met: volume between 0.2 and 0.6 mL; sperm concentration of ≥ 4

$\times 10^9$ spermatozoa/mL; sperm motility $\geq$ 80%; and $\leq$ 10% abnormal morphology. Instantly after the initial assessment, semen samples were pooled (Najafi et al., 2019c).

Extender Preparation and Semen Cryopreservation Procedure

The basic extender composition used in this study is shown in Table 1 (Moghbeli et al., 2016). Sperm samples from treated roosters were diluted with modified Beltsville in their groups in order to aspirate into 0.25 mL French straws (IMV, L'Aigle, France) to get a definitive concentration of 400×10^6 spermatozoa/mL (100×10^6 spermatozoa per straw). After that, the straws were sealed with polyvinyl alcohol powder and stored at 4°C for 3 h .The straws were placed in 4 cm over the liquid nitrogen for approximately 7 min in a 40 × 20 × 20 cm cryobox with 8,000 cm^3 liquid nitrogen. Afterwards, liquid nitrogen was used to store the straws at –196°C (Najafi et al., 2014). After a month, the frozen straws were thawed separately at 37°C for 30 s in a water bath and then evaluated individually.

Astaxanthin Nanoemulsion Preparation

In order to prevent the light sensitivity effect of Ast, the nanoemulsion was prepared with minimal exposure to light. The oil-in-water red color Ast emulsion was prepared using ultrapure water as the continuous phase and palm olein containing Ast as the disperse phase. Ast extract was first mixed with pure palm olein (15% w/w). The solution was then mixed with purified water (82.5% w/w) containing Tween 80 and lecithin as the emulsifier (2.5% w/w). The premix was homogenized using high shear homogenizer (Silverson Homogenizer, UK) at 3,000 rpm for 5 minu followed by high pressure homogenizer (model APV 1000, APV Systems, Albertsland, Denmark).

Assessment of Fresh and Post-Thawed Sperm

Fresh and post-thawed motion characteristics of sperm were analyzed by computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA) with settings programmed to detect rooster sperm (30 frames acquired, minimum contrast = 50, minimum average path velocity (**VAP**) = 5 μm/s, minimum straight line velocity (**VSL**) = 6 μm/s, non-motile head size = 7 pix and nonmotile head intensity = 95) (Mocé et al., 2010). A prewarmed (37°C) Leja chamber (Leja 4, Leja Products Luzernestraat B.V., Holland) was inserted under the thermal plate of the microscope, and then the semen from the 3 straws in the chamber was assessed individually. Five fields, randomly selected by the computer and estimated for each semen sample (Najafi et al., 2019b), and 9 motility parameters were recorded using a phase-contrast microscope (CKX41; Olympus, Tokyo, Japan), including total motile sperm (**TM**, %), progressive motile sperm (**PM**, %), average path velocity (**VAP**, μm/s), straight linear velocity (**VSL**, μm/s), curvilinear velocity (**VCL**, μm/s), amplitude of lateral head displacement (**ALH**, μm), straightness (**STR**, %), and linearity (**LIN**, %).

Sperm Viability

Sperm viability was evaluated using the eosin-nigrosin stain method (Safari Asl et al., 2018). To prepare the sperm suspension smears, a drop of sperm sample (10 μL) was mixed with 2 drops (20 μL) of stain on a prewarmed slide and immediately expanded with another slide. After drying, the viable and non-viable sperm on the slide were assessed by counting 200 cells at ×400 magnification under phase-contrast microscopy (CKX41; Olympus). Sperm with partial or complete purple staining were considered non-viable, and only cells that showed complete exclusion of stain were considered viable.

Sperm Morphology

Thirty microliters of semen was placed in 1 mL Hancock's solution to evaluate total sperm abnormalities (Mehdipour et al., 2018). This solution included 62.5 mL formalin (with 37% formaldehyde), PBS buffer (150 mL), sodium saline solution (150 mL), and double-distilled water (500 mL). To specify acrosome, head, and tail abnormalities, 10 μL of processed sperm was handled on a slide. The abnormalities percentage was determined by counting a total number of 300 sperm with a phase-contrast microscope (×1,000 magnification; oil immersion).

Plasma Membrane Integrity Evaluation

The hypoosmotic swelling test was used to evaluate the functionality of sperm plasma membrane after freeze-thawing (Bazyar et al., 2019). This test was performed by placing 5 mL of extended semen in a 50 μL hypoosmotic solution with 100 mOsm/l (19.2 mM sodium citrate and 57.6 mM fructose). After incubation for 20 min, the sperm was verified under a phase-contrast microscope (×1,000 magnification), and at least 300 spermatozoa with swollen and non-swollen tails were recorded.

Lipid Peroxidation

The malondialdehyde (**MDA**) level, as indicative of LPO in the sperm membrane phospholipids, was measured using the thiobarbituric acid (**TBA**) reaction (Feyzi et al., 2018). Briefly, 1 mL of diluted sperm (250×10^6 mL^{-1}) was added to 1 mL of cold 20% (w/v) TBA to precipitate protein. The precipitate was centrifuged (960 × g 15 min), and 1 mL of 0.67% (w/v) TBA was incubated with the supernatant (1 mL) in a boiling water (100°C) bath for 10 min. After cooling, a

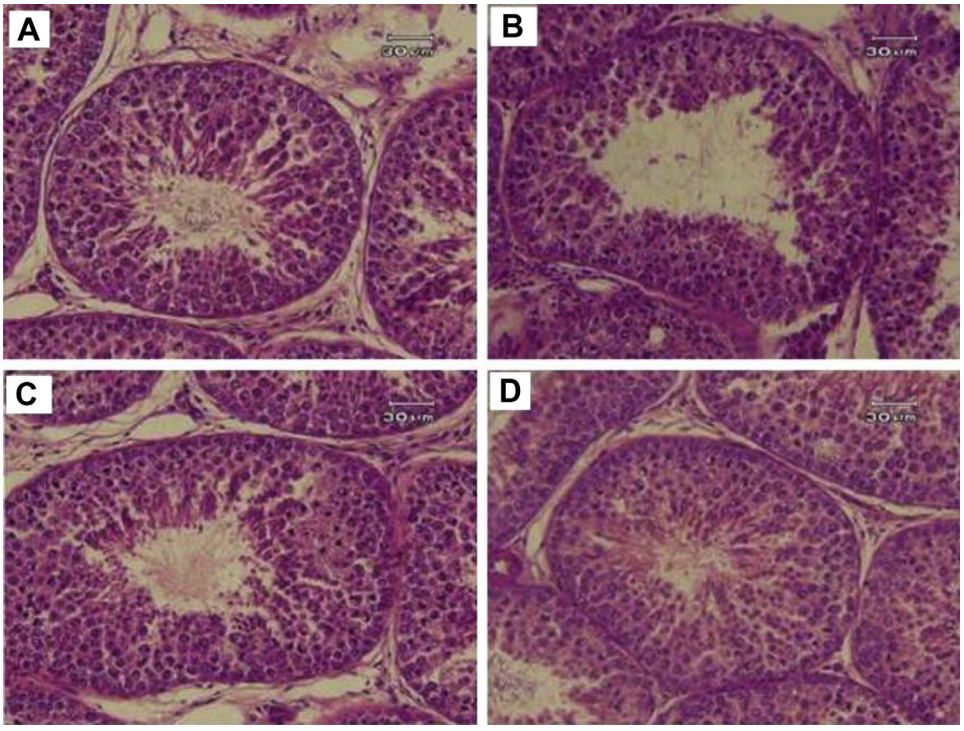

Figure 1. HE staining results of testis. The testes of the control group showed different stages of spermatogenesis (A). The Cd group showed pyknosis undergoing necrosis of cells. There were also fewer Leydig cells (B). Ast-treated group increased sperm cell proliferation and improved seminiferous tubule structure (C). The Cd+Ast-treated group increased sperm cell proliferation and maturation as well as the number of Leydig cells in the seminiferous tubule and also the structure of the seminiferous tubule was more complete, and closely connected with other tubules (D). 400X.

spectrophotometer (UV-1200, Shimadzu, Japan) was used to read the absorbance at 532 nm. All MDA values were reported as nmol/mL.

Superoxide Dismutase and Total Antioxidant Capacity Levels

The supernatant absorbances were recorded by a spectrophotometer (UV-1200, Shimadzu, Japan) at 532 nm. The SOD value was recorded according to the adapted procedure explained elsewhere. In this reaction, superoxide anions cause pyrogallol oxidation. Pyrogallol oxidation inhibition by SOD was followed by using a spectrophotometer at 420 nm. TAC was measured by monitoring hydrogen peroxide decomposition.

GPx Activity Assessment

The GPx value was determined according to Najafi et al. (2018b). The reaction substance consisted of potassium phosphate buffer (50 mM, pH 7.0), EDTA (1 mM), 1 mM sodium azide (NaN_3), 0.2 mM decreased nicotinamide adenine dinucleotide phosphate (**NADPH**), glutathione reductase (1 EU mL^{-1}) and 1 mM GSH. The sperm sample (0.1 mL) was blended with 0.8 mL of the reaction substance, incubated at 25°C for 5 min before the beginning of the reaction, and induced by the addition of 0.1 mL of a H_2O_2 solution (final H_2O_2 dose: 0.25 mM). A spectrophotometer (UV-1200, Shimadzu, Japan) was used to read absorbance at 412 nm in 5 min. GPx activity was expressed as international units (IU) per g protein.

Histology of the Testes

The roosters were killed at the end of the experiment, and the testes were isolated and fixed in Bouin's fixator. The samples were removed from fixative and dehydrated using a graded series of ethanol (50, 70, 80, 90, 96, and 100%), cleared in xylene, and embedded in paraffin. The paraffin sections were sliced using a microtome. The 5 μm microscopic sections were prepared, and at least 2 slides from each testis were stained with hematoxylin and eosin for histological assessment.

Statistical Analysis

The experiment was performed in 5 replicates. Data were checked for normal distribution using a univariate procedure and the Shapiro–Wilk test. Arc sine (for percentage data) transformation of data was used when appropriate. Single and repeated measurements data were analyzed by PROC GLM and PROC Mixed SAS 9.1 (version 9.1, 2002; SAS institute Cary, NC), respectively. The results are presented as the Lsmean ± SEM. The Tukey test was applied to compare Lsmeans, and the level of significance was adjusted to $P < 0.05$.

Table 2. Effect of different antioxidants on motion parameters of fresh and post-thawed rooster sperm assessed by CASA (mean ± SEM).

	Treatment									
	Fresh					Post-thawed				
Parameter	Control	Cd	Ast	Cd + Ast	SEM	Control	Cd	Ast	Cd + Ast	SEM
MOT (%)	91.32^{a}	80.61^{b}	92.27^{a}	88.36^{a}	1.23	57.70^{b}	37.29^{c}	68.67^{a}	54.67^{b}	2.46
PROG (%)	51.22^{b}	39.61^{c}	57.74^{a}	50.45^{b}	1.34	13.97^{b}	5.84^{c}	17.67^{a}	13.25^{b}	0.47
VAP (μm/s)	38.21^{b}	30.11^{c}	42.95^{a}	36.41^{b}	0.88	28.58^{b}	19.49^{d}	33.30^{a}	26.10^{c}	0.47
VSL (μm/s)	24.74^{b}	19.42^{c}	28.92^{a}	23.14^{b}	0.74	15.91^{b}	12.68^{c}	19.26^{a}	16.36^{b}	0.64
VCL (μm/s)	63.09	60.21	64.39	62.24	2.14	56.51	51.13	58.19	54.53	1.8
LIN (%)	38.13^{a,b}	30.1^{b}	43.8^{a}	36.7^{a,b}	1.94	28.28^{a,b}	24.93^{b}	33.35^{a}	30.20^{a,b}	1.75
STR (%)	65.42	61.04	67.89	64.24	2.32	55.72	65.22	57.85	62.71	2.48
ALH (μm)	4.13	3.94	4.20	4.01	0.43	2.96	2.65	3.44	3.13	0.51
BCF (Hz)	19.07	17.05	19.8	18.9	1.32	17.55	16.9	18.41	17.75	0.93

Abbreviations: MOT, total motility; PROG, progressive motility; VCL, curvilinear velocity; VAP, average path velocity; VSL, straight-line velocity; LIN, linearity; STR, straightness; ALH, amplitude of lateral head displacement; BCF, beat/cross frequency.

Different letters exhibit differences among treatment groups ($P < 0.05$).

RESULTS

Histological Result

The testes of the control group showed different stages of spermatogenesis (Figure 1A). Compared with the control group (Figure 1A), the Cd group showed pyknosis undergoing necrosis of cells (Figure 1B). There were also fewer Leydig cells (Figure 1B). No significant differences were seen between the control and Ast groups (Figure 1C). Compared with the Cd group, the Cd+Ast-treated group increased sperm cell proliferation and maturation as well the number of Leydig cells in the seminiferous tubule and also the structure of the seminiferous tubule was more complete, and closely connected with other tubules (Figure 1D).

Motility and Kinetic Parameters

Table 2 shows data for the effect of Cd and Ast NPs on sperm motility variables under the following conditions: when the rooster sperm was fresh and then after freezing-thawing. Cd (1 mg/100 g/body SC) resulted in significantly ($P < 0.05$) lower total (80.61 ± 1.23) and progressive (39.61 ± 1.34) motility, and it is restored to similar control values after treatment with Ast (group Cd + Ast; 88.36 ± 1.23 and 50.45 ± 1.34, respectively). Post-thawed total (37.29 ± 2.46) and progressive (5.84 ± 0.47) motility showed the same patterns, also restoring to similar values relative with control in Ast + Cd group (54.67 ± 2.46 and 13.25 ± 0.47, respectively). In addition, post-thawing values using Ast NPs (25 mg/kg p.o) resulted in significantly ($P < 0.05$) higher total (68.76 ± 2.46) and progressive (17.67 ± 0.47) motility compared to other groups. Moreover, the VAP (fresh: 42.95 ± 0.88 and post-thawed: 33.30 ± 0.47) and VSL (fresh: 28.92 ± 0.74 and post-thawed: 19.26 ± 0.64) values in Ast NPs group were significantly ($P < 0.05$) higher than the other groups. No significant differences were observed in LIN, STR, ALH, and BCF in the treatment groups compared to the control.

Viability, Membrane Integrity, and Sperm Abnormalities

The influence of Cd and Ast NPs on sperm viability, membrane integrity, and abnormal forms is presented in Table 3. Post-thawed, higher sperm viability and membrane integrity (75.76 ± 1.86, 50.43 ± 1.54; respectively) were found in the Ast NPs group compared to the others, but not in fresh sperm, where only membrane integrity was higher (87.48 ± 1.39).The percentage of abnormal forms decreased both in fresh (4.90 ± 0.51) and post-thawed (17.01 ± 1.25) sperm in Ast group compared with the rest of the groups, including control. Moreover, Cd induced lower ($P < 0.05$) viability (fresh: 82.4 ± 1.57 and post-thawed: 43.56 ± 1.86) and integrity (fresh: 72.31 ± 1.39 and post-thawed: 31.87 ± 1.54) and higher abnormal forms (fresh: 9.81 ± 0.51 and post-thawed: 37.12 ± 1.25) among the control and treatment groups.

Oxidative Variables

The effects of Cd and Ast NPs on the oxidative variables status of rooster sperm after freeze-thawing are summarized in Table 4. There were no differences in the MDA value in the control group as compared with the other groups in post-thawed values. However, fresh values of MDA were significantly higher in Cd group compared with the control and treatment groups. Furthermore, the lowest values for CAT, TAC, and GPX activity (fresh: 29.17 ± 3.51, 0.66 ± 0.12, 42.80 ± 1.63 and post-thawed: 32.31 ± 3.27, 0.79 ± 0.21, 48.20 ± 1.50; respectively) were obtained in the Cd group. In general, addition of Ast NPs to the roosters' diet in the Cd group (Cd + Ast) improved CAT ($P < 0.05$), TAC, and GPX activity (fresh: 45.23 ± 3.51, 1.66 ± 0.12, 51.47 ± 1.63 and post-thawed: 48.80 ± 3.27, 1.71 ± 0.21, 55.34 ± 1.50; respectively) compared to the Cd group. However, there were no differences in SOD activity among the control and treatment groups.

Table 3. Percentage of viability, membrane integrity, and abnormal forms of fresh and post-thawed rooster sperm (mean ± SEM).

	Treatment									
	Fresh					Post-thawed				
Parameter	Control	Cd	Ast	Cd + Ast	SEM	Control	Cd	Ast	Cd + Ast	SEM
Viability (%)	92.01^{a}	82.4^{b}	94.30^{a}	90.36^{a}	1.57	64.69^{b}	43.56^{c}	75.76^{a}	57.24^{b}	1.86
Membrane integrity (%)	82.23^{b}	72.31^{c}	87.48^{a}	81.34^{b}	1.39	40.04^{b}	31.87^{c}	50.43^{a}	41.91^{b}	1.54
Abnormal forms (%)	6.59^{b}	9.81^{a}	4.90^{c}	7.21^{b}	0.51	25.67^{b}	37.12^{a}	17.01^{c}	30.15^{b}	1.25

Different letters exhibit differences among treatment groups ($P < 0.05$).

Testis/Body Weight Ratio

Results for the testis/body weight (g/kg) ratio from treated roosters and controls are shown in Table 5. Cd-treated roosters showed a significant ($P < 0.05$) decrease in testis weight (g) compared to the control group (38.45 ± 0.08, 41.30 ± 0.08, respectively). Using Ast NPs reversed ($P < 0.05$) Cd induced testicular atrophy (Figure 1B) in Cd + Ast NPs group. However, there were no differences in body weight and testis/body weight ratio among the control and treatment groups.

DISCUSSION

The main aim of this experiment was to study the effect of Ast NPs antioxidant activity and also the combined effect on an induced model by Cd exposure on testis weight, ROS generation, quality parameters, and the oxidative status of fresh and cryopreserved rooster sperm. In this study, we also determined Ast NPs antioxidant activity against Cd-induced oxidative stress by assessing motility parameters and oxidative variables in fresh and post-thawed rooster sperm. The improvement of semen characteristics in fresh semen seemed to be a result of the dietary supplementation of antioxidants. The findings of the present study show that the results of fresh and cryopreserved sperm are in consistency.

Nanotechnology is a rapidly progressing field. Nanoparticles hold tremendous potential as an effective drug delivery system. Nanosystems with different compositions and biological properties have been extensively investigated for drug delivery applications (Koo et al., 2005; Najafi et al., 2018a). In this study, we demonstrated that dietary Ast NPs have a protective effect against Cd-induced LPO and can improve sperm quality that has decreased during Cd exposure (administrated subcutaneously).

The antioxidant defense system plays a key role in protecting the male reproductive system against oxidative damage (Yang et al., 2016). It has been reported that food supplementation with Ast in 30 infertile men resulted in a significant reduction in seminal ROS and improved sperm motility and morphology (Comhaire et al., 2005). Another study documented that Ast treatment significantly restored sperm DNA damage induced by cyclophosphamide and showed a protective effect against cyclophosphamide-induced testicular toxicity confirmed by testes histology in mice (Tripathi and Jena, 2008).

Our findings in the present study showed that diet supplementation with Ast NPs enhanced sperm quality in the Ast group compared to the control. Furthermore, we proved that Ast NPs in the diet could decrease the detrimental effect of Cd in sperm motility, viability, and oxidative status. In another study, it was noted that treatment of rats with CoQ10 and Vit E alone and/or in combination with Cd decreased the toxic effect of Cd on antioxidant enzyme activity (Ognjanović et al., 2010). In the present study, Ast NPs reduced cellular toxicity caused by Cd-induced ROS and protected the post-thawing sperm samples in the anti-oxidative system.

One of the reported roles of carotenoids is their antioxidant role in animals. Oxidative stress is of great concern in poultry industries with breeder roosters. Normally, various antioxidants and enzymes in seminal plasma protect sperm from oxidative attack (Min et al., 2016). The efficiency of dietary carotenoid supplementation on antioxidant status has been investigated and shown to rescue various cells and tissues from oxidative damage (Pulido et al., 2000; Wang et al., 2006). Ast is a naturally

Table 4. Malondialdehyde (MDA) concentration and antioxidant enzyme activity status of rooster sperm after fresh and freeze-thawing (mean ± SEM).

	Treatment									
	Fresh					Post-thawed				
Parameter	Control	Cd	Ast	Cd + Ast	SEM	Control	Cd	Ast	Cd + Ast	SEM
MDA (nmol/mL)	1.72^{b}	2.69^{a}	1.13^{c}	1.84^{b}	0.26	2.02^{a,b}	3.14^{a}	1.78^{b}	2.54^{a,b}	0.31
CAT (U/mL)	53.14^{b}	29.17^{c}	59.66^{a}	45.23^{b}	3.51	61.00^{a,b}	32.31^{c}	69.51^{a}	48.80^{b}	3.27
SOD (U/mL)	88.42	74.34	98.45	87.90	9.88	96.02	85.13	117.42	98.37	10.3
TAC (mmol/l)	1.73^{b}	0.66^{c}	2.10^{a}	1.66^{b}	0.12	1.86^{a}	0.79^{b}	2.31^{a}	1.71^{a}	0.21
GPX (IU/g protein)	54.11^{b}	42.80^{c}	59.73^{a}	51.47^{b}	1.63	58.47^{a,b}	48.20^{c}	63.46^{a}	55.34^{b}	1.5

Different letters exhibit differences among treatment groups ($P < 0.05$).

Table 5. Testis/body weight (g/kg) ratio of treated roosters and controls at 5 wk following treatment (birds at 25 wk old).

Treatment	Testis weight (g)	Live body weight (kg)	Testis/body weight (g/kg)
Control	41.30[a]	4.07	10.16
Cd	38.45[b]	3.85	10.03
Ast	45.95[c]	4.12	11.17
Cd + Ast	39.93[d]	3.92	10.19
SEM	0.08	0.10	0.28

Different letters exhibit differences among treatment groups ($P < 0.05$).

occurring carotenoid with strong antioxidant properties, which shows low oral bioavailability due to its lipophilicity. The use of nano emulsion-based formulations is one of several approaches found to be efficient in improving the bioavailability of lipophilic entities such as Ast (Affandi et al., 2011).

Ast NPs (25 mg/kg) seemed to be an effective dose for enhancing sperm total and progressive motility, vitality and membrane integrity as outcomes improved relative to the controls. Furthermore, our results confirmed that oral treatment with Ast NPs in rooster could counteract the detrimental effect of Cd in sperm motility, morphological appearance, and oxidative status indexes. These results are in agreement with Ognjanović et al. (2010), who demonstrated that treatment with CoQ10 and Vit E reversed Cd-induced alterations in the antioxidant defense system and significantly prevented Cd-induced testes damage.

Heavy metal toxicity has become a serious global public health problem. With the rapid development of modern industries, reproductive impairment caused by environmental pollution should never be neglected. Many experiments have used Ast to enhance sperm quality in human (Dona et al., 2013; Andrisani et al., 2015), ram (Fang et al., 2015), fish (Mansour et al., 2006; Tizkar et al., 2015a, b), and rat (Vahidinia et al., 2017) and reported that this antioxidant can improve sperm capacitation. In this study, we investigated the protective effects of Ast against LPO, and changes in enzymatic (MDA, CAT, SOD, TAC, and GPX) defense systems induced by Cd in rooster sperm. In our study, by examining sperm anti-oxidative variables, we showed a reduction in MDA and an increase in CAT, TAC, and GPX concentrations in the Cd + Ast NPs group compared to the Cd group, which was the most definitive indicator of Ast-mediated antioxidant protection and reduction in sperm LPO.

Data collected from the histological examination of seminiferous tubules also confirmed seminiferous cell depletion in the Cd-treated group and increased sperm cell proliferation and improved seminiferous tubule structure in the Cd + Ast NPs group compared to the Cd group.

In conclusion, Ast NPs (25 mg/kg p.o) improved cryosurvival and enhanced the preservation of sperm motility, membrane integrity, and viability. Also, the results suggest that Cd, at the concentration used in our experimental approach, may increase LPO and alter the antioxidant defense systems, resulting in oxidative damage to rooster sperm quality parameters. Combined treatment with Ast NPs and Cd showed protective effects against LPO and changes in enzymatic (MDA, CAT, SOD, TAC, and GPX) antioxidant systems in rooster sperm. The results of our work suggest that Ast NPs at 25 mg/kg may act as a potent antioxidant in protecting rooster testes against oxidative stress induced by Cd. The present study suggests that Cd impairs the reproductive function in male Ross 308 breeder roosters through toxic effects on the testis.

ACKNOWLEDGMENTS

The authors would like to thank Baqiyatallah University of Medical Sciences and the deputy of clinical development medical center of Baqiyatallah hospital for their helps.

REFERENCES

Acosta, I. B., A. S. V Junior, E. S. EF, T. F. Cardoso, J. S. Caldas, R. D. Jardim, and C. D. Corcini. 2016. Effects of exposure to cadmium in sperm cells of zebrafish, Danio rerio. Toxicol. Rep. 3:696–700.

Affandi, M. M. M., T. Julianto, and A. Majeed. 2011. Development and stability evaluation of astaxanthin nanoemulsion. Asian J. Pharm. Clin. Res. 4:142–148.

Alkhedaide, A., Z. S. Alshehri, A. Sabry, T. Abdel-Ghaffar, M. M. Soliman, and H. Attia. 2016. Protective effect of grape seed extract against cadmium-induced testicular dysfunction. Mol. Med. Rep. 13:3101–3109.

Andrisani, A., G. Dona, E. Tibaldi, A. M. Brunati, C. Sabbadin, D. Armanini, G. Alvisi, S. Gizzo, G. Ambrosini, E. Ragazzi, and L. Bordin. 2015. Astaxanthin improves human sperm capacitation by inducing lyn displacement and activation. Mar. Drugs 13:5533–5551.

Azin, E., H. Moghimi, and R. A. Taheri. 2017. Development of carbon nanotube-mycosorbent for effective Congo red removal: optimization, isotherm and kinetic studies. Desalin. Water Treat. 94:222–230.

Bagdeli, S., A. H. Rezayan, R. A. Taheri, M. Kamali, and M. Hosseini. 2017. FRET-based immunoassay using CdTe and AuNPs for the detection of OmpW antigen of Vibrio cholerae. J. Lumin. 192:932–939.

Bazyar, M., M. Sharafi, and A. Shahverdi. 2019. Changes in seminal parameters and hormonal profile with use of aromatase inhibitor in management of aging broiler breeder roosters. Poult. Sci. 98:6100–6107.

Benoff, S., K. Auborn, J. L. Marmar, and I. R. Hurley. 2008. Link between low-dose environmentally relevant cadmium exposures and asthenozoospermia in a rat model. Fertil. Steril. 89:e73–e79.

Burrows, W. H., and J. P. Quinn. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poult. Sci. 16:19–24.

Cerolini, S., K. A. Kelso, R. C. Noble, B. K. Speake, F. Pizzi, and L. G. Cavalchini. 1997. Relationship between spermatozoan lipid composition and fertility during aging of chickens. Biol. Reprod. 57:976–980.

Cerolini, S., L. Zaniboni, A. Maldjian, and T. Gliozzi. 2006. Effect of docosahexaenoic acid and alpha-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to peroxidation. Theriogenology 66:877–886.

Comhaire, F. H., Y. El Garem, A. Mahmoud, F. Eertmans, and F. Schoonjans. 2005. Combined conventional/antioxidant "Astaxanthin" treatment for male infertility: a double blind, randomized trial. Asian J. Androl. 7:257–262.

De Souza Predes, F., M. A. S. Diamante, and H. Dolder. 2010. Testis response to low doses of cadmium in Wistar rats. Int. J. Exp. Pathol. 91:125–131.

Dian, L., Z. Yang, F. Li, Z. Wang, X. Pan, X. Peng, X. Huang, Z. Guo, G. Quan, and X. Shi. 2013. Cubic phase nanoparticles for sustained release of ibuprofen: formulation, characterization, and enhanced bioavailability study. Int. J. Nanomed. 8:845.

Dona, G., I. Kozuh, A. M. Brunati, A. Andrisani, G. Ambrosini, G. Bonanni, E. Ragazzi, D. Armanini, G. Clari, and L. Bordin. 2013. Effect of astaxanthin on human sperm capacitation. Mar. Drugs 11:1909–1919.

Fang, Y., R. Zhong, L. Chen, C. Feng, H. Sun, and D. Zhou. 2015. Effects of astaxanthin supplementation on the sperm quality and antioxidant capacity of ram semen during liquid storage. Small Rumin. Res. 130:178–182.

Fasihi-Ramandi, M., H. Ghobadi-Ghadikolaee, S. Ahmadi-Renani, R. A. Taheri, and K. Ahmadi. 2018. Vibrio cholerae lipopolysaccharide loaded chitosan nanoparticle could save life by induction of specific immunoglobulin isotype. Artif. Cells Nanomed. Biotechnol. 46:56–61.

Fattah, A., M. Sharafi, R. Masoudi, A. Shahverdi, V. Esmaeili, and A. Najafi. 2017. l-Carnitine in rooster semen cryopreservation: flow cytometric, biochemical and motion findings for frozen-thawed sperm. Cryobiology 74:148–153.

Feyzi, S., M. Sharafi, and S. Rahimi. 2018. Stress preconditioning of rooster semen before cryopreservation improves fertility potential of thawed sperm. Poult. Sci. 97:2582–2590.

Hajipour, H., H. Hamishehkar, S. Nazari Soltan Ahmad, S. Barghi, N. F. Maroufi, and R. A. Taheri. 2018. Improved anticancer effects of epigallocatechin gallate using RGD-containing nanostructured lipid carriers. Artif. Cells Nanomed. Biotechnol. 46:283–292.

Jamalan, M., M. A. Ghaffari, P. Hoseinzadeh, M. Hashemitabar, and M. Zeinali. 2016. Human sperm quality and metal toxicants: protective effects of some flavonoids on male reproductive function. Int. J. Fertil. Steril. 10:215–223.

Khansary, M. A., M. Mellat, S. H. Saadat, M. Fasihi-Ramandi, M. Kamali, and R. A. Taheri. 2017. An enquiry on appropriate selection of polymers for preparation of polymeric nanosorbents and nanofiltration/ultrafiltration membranes for hormone micropollutants removal from water effluents. Chemosphere 168:91–99.

Koo, O. M., I. Rubinstein, and H. Onyuksel. 2005. Role of nanotechnology in targeted drug delivery and imaging: a concise review. Nanomed. Nanotechnol. Biol. Med. 1:193–212.

Lai, J.-P., Y. Jiang, X.-W. He, J.-C. Huang, and F. Chen. 2004. Separation and determination of astaxanthin from microalgal and yeast samples by molecularly imprinted microspheres. J. Chromatogr. B 804:25–30.

Li, Y., J. Wu, W. Zhou, and E. Gao. 2016. Association between environmental exposure to cadmium and human semen quality. Int. J. Environ. Health Res. 26:175–186.

Mansour, N., M. A. McNiven, and G. F. Richardson. 2006. The effect of dietary supplementation with blueberry, alpha-tocopherol or astaxanthin on oxidative stability of Arctic char (Salvelinus alpinus) semen. Theriogenology 66:373–382.

Massanyi, P., J. Weis, N. Lukac, J. Trandzik, and J. Bystricka. 2008. Cadmium, zinc, copper, sodium and potassium concentrations in rooster and turkey semen and their correlation. J. Environ. Sci. Heal. Part A 43:563–565.

Mehdipour, M., H. Daghigh Kia, G. Moghaddam, and H. Hamishehkar. 2018. Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. Theriogenology 116:89–94.

Mehdipour, M., H. D. Kia, M. Nazari, and A. Najafi. 2017. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. Cryobiology 78:34–40.

Miki, W. 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. 63:141–146.

Min, Y., T. Sun, Z. Niu, and F. Liu. 2016. Vitamin C and vitamin E supplementation alleviates oxidative stress induced by dexamethasone and improves fertility of breeder roosters. Anim. Reprod. Sci. 171:1–6.

Mocé, E., I. Grasseau, and E. Blesbois. 2010. Cryoprotectant and freezing-process alter the ability of chicken sperm to acrosome react. Anim. Reprod. Sci. 122:359–366.

Moghbeli, M., H. Kohram, A. Zare-Shahaneh, M. Zhandi, M. Sharafi, M. M. Nabi, V. Zahedi, and H. Sharideh. 2016. Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? Cryobiology 72:264–268.

Najafi, A., H. Daghigh Kia, M. Mehdipour, M. Shamsollahi, and D. J. Miller. 2019a. Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm? Cryobiology 87:47–51.

Najafi, A., H. D. Kia, H. Hamishehkar, G. Moghaddam, and S. Alijani. 2019b. Effect of resveratrol-loaded nanostructured lipid carriers supplementation in cryopreservation medium on post-thawed sperm quality and fertility of roosters. Anim. Reprod. Sci. 201:32–40.

Najafi, A., M. H. Najafi, Z. Zanganeh, M. Sharafi, F. Martinez-Pastor, and H. Adeldust. 2014. Cryopreservation of ram semen in extenders containing soybean lecithin as cryoprotectant and hyaluronic acid as antioxidant. Reprod. Domest. Anim. 49:934–940.

Najafi, A., R. A. Taheri, M. Mehdipour, G. Farnoosh, and F. Martínez-Pastor. 2018a. Lycopene-loaded nanoliposomes improve the performance of a modified Beltsville extender broiler breeder roosters. Anim. Reprod. Sci. 195:168–175.

Najafi, A., R. A. Taheri, M. Mehdipour, F. Martínez-Pastor, A. A. Rouhollahi, and M. R. Nourani. 2019c. Improvement of post-thawed sperm quality in broiler breeder roosters by ellagic acid-loaded liposomes. Poult. Sci. 98:440–446.

Najafi, D., R. A. Taheri, A. Najafi, A. A. Rouhollahi, and M. Alvarez-Rodriguez. 2018b. Effect of Achillea millefolium-loaded nanophytosome in the post-thawing sperm quality and oxidative status of rooster semen. Cryobiology 82:37–42.

Ognjanović, B. I., S. D. Marković, N. Z. Đorđević, I. S. Trbojević, A.Š.. Štajn, and Z. S. Saičić. 2010. Cadmium-induced lipid peroxidation and changes in antioxidant defense system in the rat testes: protective role of coenzyme Q 10 and Vitamin E. Reprod. Toxicol. 29:191–197.

Partyka, A., W. Nizanski, J. Bajzert, E. Lukaszewicz, and M. Ochota. 2013. The effect of cysteine and superoxide dismutase on the quality of post-thawed chicken sperm. Cryobiology 67:132–136.

Pulido, R., L. Bravo, and F. Saura-Calixto. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem. 48:3396–3402.

Rad, H. M., M. Eslami, and A. Ghanie. 2016. Palmitoleate enhances quality of rooster semen during chilled storage. Anim. Reprod. Sci. 165:38–45.

Rasaie, S., S. Ghanbarzadeh, M. Mohammadi, and H. Hamishehkar. 2014. Nano phytosomes of quercetin: a promising formulation for fortification of food products with antioxidants. Pharm. Sci. 20:96.

Riaz, M., Z. Mahmood, M. Shahid, M. U. Saeed, I. M. Tahir, S. A. Shah, N. Munir, and A. El-Ghorab. 2016. Impact of reactive oxygen species on antioxidant capacity of male reproductive system. Int. J. Immunopathol. Pharmacol. 29:421–425.

Safari Asl, R., F. Shariatmadari, M. Sharafi, M. A. Karimi Torshizi, and A. Shahverdi. 2018. Improvements in semen quality, sperm fatty acids, and reproductive performance in aged Ross breeder roosters fed a diet supplemented with a moderate ratio of n-3: n-6 fatty acids. Poult. Sci. 97:4113–4121.

Sayed, M. M., K. M. A. Hassanein, and W. Senosy. 2014. Protective effects of thymoquinone and l-cysteine on cadmium-induced reproductive toxicity in rats. Toxicol. Rep. 1:612–620.

Siu, E. R., D. D. Mruk, C. S. Porto, and C. Y. Cheng. 2009. Cadmium-induced testicular injury. Toxicol. Appl. Pharmacol. 238:240–249.

Sood, S., N. Jawahar, K. Jain, K. Gowthamarajan, and S. Nainar Meyyanathan. 2013. Olanzapine loaded cationic solid lipid nanoparticles for improved oral bioavailability. Curr. Nanosci. 9:26–34.

Swami, D. S., P. Kumar, R. K. Malik, M. Saini, D. Kumar, and M. H. Jan. 2017. Cysteamine supplementation revealed detrimental effect on cryosurvival of buffalo sperm based on computer-assisted semen analysis and oxidative parameters. Anim. Reprod. Sci. 177:56–64.

Tandon, S. K., S. Singh, S. Prasad, K. Khandekar, V. K. Dwivedi, M. Chatterjee, and N. Mathur. 2003. Reversal of cadmium induced oxidative stress by chelating agent, antioxidant or their combination in rat. Toxicol. Lett. 145:211–217.

Tizkar, B., R. Kazemi, A. Alipour, A. Seidavi, G. Naseralavi, and J. T. Ponce-Palafox. 2015. Effects of dietary supplementation with astaxanthin and beta-carotene on the semen quality of goldfish (Carassius auratus). Theriogenology 84:1111–1117.

Tripathi, D. N., and G. B. Jena. 2008. Astaxanthin inhibits cytotoxic and genotoxic effects of cyclophosphamide in mice germ cells. Toxicology 248:96–103.

Tvrda, E., Z. Knazicka, and N. Lukac. 2012. Selected heavy metals versus antioxidant parameters in bull seminal plasma - a comparative study. J. Environ. Sci Health A Tox. Hazard Subst. Environ. Eng. 47:1261–1266.

Tvrda, E., Z. Knazicka, J. Lukacova, M. Schneidgenova, Z. Goc, A. Gren, C. Szabo, P. Massanyi, and N. Lukac. 2013. The impact of lead and cadmium on selected motility, prooxidant and antioxidant parameters of bovine seminal plasma and spermatozoa. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 48:1292–1300.

Vahidinia, A., A. R. Rahbar, and M. M. Shakoori Mahmoodabadi. 2017. Effect of astaxanthin, vitamin E, and vitamin C in combination with calorie restriction on sperm quality and quantity in male rats. J. Diet Suppl. 14:252–263.

Wang, H. F., M. Chang, T. T. Peng, Y. Yang, N. Li, T. Luo, Y. M. Cheng, M. Z. Zhou, X. H. Zeng, and L. P. Zheng. 2017. Exposure to cadmium impairs sperm functions by reducing catsper in mice. Cell. Physiol. Biochem. 42:44–54.

Wang, Y.-J., Y.-H. Chien, and C.-H. Pan. 2006. Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins, Hyphessobrycon callistus. Aquaculture 261:641–648.

Yang, S. H., M. Long, L. H. Yu, L. Li, P. Li, Y. Zhang, Y. Guo, F. Gao, M. D. Liu, and J. B. He. 2016. Sulforaphane prevents testicular damage in kunming mice exposed to cadmium via activation of Nrf2/ARE signaling pathways. Int. J. Mol. Sci. 17:1703.

Zhang, L., Q. Li, G. Zheng, Y. Chen, M. Huang, L. Zhang, and X. Lin. 2017. Protective effect of Lycium barbarum polysaccharides against cadmium-induced testicular toxicity in male mice. Food Funct 8:2322–2330.

Zhou, Y., X. M. Fu, D. L. He, X. M. Zou, C. Q. Wu, W. Z. Guo, and W. Feng. 2016. Evaluation of urinary metal concentrations and sperm DNA damage in infertile men from an infertility clinic. Environ. Toxicol. Pharmacol. 45:68–73.