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Alpha-zearalenol negatively influences ram sperm parameters during liquid storage

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Abstract

This study was aimed to investigate the effects of 17 β-estradiol (E2) and α-zearalenol (α-ZOL) on motility parameters, plasma membrane integrity, levels of produced nitric oxide (NO) and total antioxidant capacity of Ghezel ram sperm during the liquid storage at 4 ˚C, for various periods of time. Semen samples were collected from four rams and diluted with Tris–egg yolk extender and supplemented with E2 (100 µmol) or different concentrations of α-ZOL (100 pmol, 100 nmol and 100 µmol) at a final concentration of 200 × 10^6 sperm per mL. We failed to show any significant effect of E2 at 100 µmol concentration on ram's sperm parameters while α-ZOL resulted in a significant decrease of plasma membrane integrity at 100 µmol concentration (55.40% for α-ZOL *vs* 62.20% for control) after 96 hr incubation. Alpha-ZOL had decreasing effect on sperm motility parameters including curvilinear velocity and average path velocity at 100 µmol concentration after 96 hr storage. Although remarkable reduction of total antioxidant capacity at high concentration of α-ZOL and long incubation time was found, however no significant changes were recorded in NO level during storage time. It was concluded that the detrimental effect of α-ZOL on ram sperm might be attributed to its induced oxidative stress and damage to the plasma membrane.

تأثیر منفی آلفا زیرالینول برروی فراسنجه‌های اسپرم قوچ طی نگهداری بصورت مایع

چکیده

این مطالعه به منظور ارزیابی اثرات اضافه کردن ۱۷بتا-استرادیول و آلفا-زیرالینول برروی تحرک، سلامت غشای پلاسمایی اسپرم قوچ، سطح نیتریک اکساید و ظرفیت کل آنتی اکسیدانی تام اسپرم قوچ در طی نگهداری بصورت مایع و در دمای چهار درجه سانتی گراد برای مدت زمان های مختلف انجام گرفت. برای این منظور نمونه منی از چهار رأس قوچ قزل جمع‌آوری و با رقیق کننده تریس-زرده تخم مرغ بدون ترکیبات استروژنی (کنترل)، حاوی ۱۰۰میکرومولار ۱۷بتا-استرادیول یا غلظت های مختلف آلفا زیرالینول (۱۰۰ پیکو مولار، ۱۰۰نانومولار و ۱۰۰میکرومولار) به میزان ۲۰۰ میلیون اسپرم در هر میلی لیتر، رقیق شدند. بر اساس نتایج بدست آمده استرادیول در غلظت ۱۰۰ میکرومول اثر معنی داری برروی هیچکدام از فراسنجه های اسپرم نداشت درصورتیکه آلفا زیرالینول در غلظت ۱۰۰ میکرومول پس از ۹۶ ساعت نگهداری باعث کاهش معنی داری در سلامت غشای پلاسمایی نسبت به گروه کنترل گردید (۵۵/۴۰ درصد برای آلفا زیرالینول در برابر ۶۲/۲۰ درصد برای گروه کنترل). آلفا زیرالینول در غلظت ۱۰۰ میکرومول پس از ۹۶ ساعت نگهداری باعث کاهش فراسنجه های حرکتی ازجمله سرعت اسپرم در مسیر واقعی و میانگین سرعت در مسیر مستقیم گردید. هرچند در غلظت های بالای آلفا زیرالینول و زمان طولانی نگهداری، کاهش مشخصی در ظرفیت آنتی اکسیدانی مشاهده گردید ولی تاثیر معنی داری برروی میزان نیتریک اکساید تولیدی در زمان های مختلف یافت نشد. بطور کلی با توجه به نتایج این تحقیق اثرات مضر آلفا زیرالینول برروی اسپرم قوچ را می توان به استرس اکسیداتیو و صدمه به غشای پلاسمایی نسبت داد.

واژه های کلیدی: آلفا-زیرالینول ، اسپرم، قوچ، مایع

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Introduction

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin that is produced by numerous species of *Fusarium* in the presence of high humidity and low temperatures (10-15 ˚C). Zearalenone exists in many cereal crops such as maize, barley, wheat, and oats as well as other foods and feeds. Zearalenone suspected to cause human diseases and hyperestrogenism in farm animals.[1] *Fusarium* is a typical field fungus infecting plants in an early stage of growth and during flowering.[2] The concentration of ZEA in food and feed varies over a wide range, depending on climatic conditions.[3] However, pre-formed ZEA is very stable, and is not destroyed by pH changes or common heat treatment during the process of drying and processing of feeds and foods.[4]

The ZEA derivatives (α- and β-zearalenol) can also be produced by *Fusarium* spp. in corn stems infected by fungi of this genus in the field.[5,6] The ZEA and some of its metabolites exhibit anabolic and growth-promoting activities in cattle and lambs.[7] Following oral exposure, ZEA is metabolized in various tissues, particularly in the liver, and its major metabolites are α- and β-zearalenol.[8-10] The more potent estrogenic active metabolite of ZEA is α-ZOL, which is naturally produced by fungi.[11,12] The effects of α-zearalenol are similar to ZEA, but α-zearalenol is generally considered for its estrogenic effects which are five- to ten- times greater than ZEA estrogenic effects.[13]

Estrogenic effects of α-zearalenol are mostly applied through the classic genomic pathway, but there are some estrogenic effects which take place by estrogens or estrogen-like compounds that are too hard to be explained by transcription of RNA and subsequent protein synthesis. There is evidence that steroid hormones/estrogen-like substances may act via pathways other than the classical genomic route.[14] The results of a recent study showed that ZEA, *α*- and *β*-ZOL quickly enhanced spontaneous contractions in lamb uterine smooth muscle in a method similar to E2, indicating non-genomic pathways for steroid actions.[15]

In vivo experiments have shown that ZEA intake can cause reproductive problems in sheep and dairy cattle. There are few reports about the toxic effects of ZEA and its derivatives on sperm parameters under *in vitro* condition, however it seems that α-ZOL affect the quantitative and qualitative parameters of ram sperm time- and dose-dependently. For example, Tsakmakidis *et al*. showed that under *in vitro* conditions, ZEA and *α*-ZOL at concentrations of 125.00, 187.50 and 250 μM lowered boar sperm viability and forward motility in a concentration- and time-dependent manner.[16] Also, in a similar study, Filannino *et al*. reported that two hour *in vitro* exposure to *α*-ZOL affected the total and progressive motility parameters of stallion sperm only when the *α*-ZOL was present at 100 μM concentration.[17]

In addition, previous studies reported negative effects of ZEA and its derivatives on boar sperm,[16] stallion sperm,[17] bovine oocytes[18] and porcine oocytes and zygotes.[19] There are no reports about the effects *α*-ZOL and E2 on ram sperm characteristics. Hence, this study was aimed to evaluate the effects of *α*-ZOL and E2 on viability, motility and plasma membrane integrity, the level of produced nitric oxide (NO) and total antioxidant capacity, during liquid storage of ram sperm at 4 ˚C for various times.

Materials and Methods

Animals and semen collection. The experimental protocols were approved by the Animal Use Committee in Urmia University, Urmia, Iran (No. 1336; 21.08.2013). Semen samples were collected from four adult and healthy Ghezel rams (3-4 years old). The rams were housed at the farm of Faculty of Agriculture, Urmia University, Urmia, Iran. During the experimental period, rams had free access to good quality hay and water was supplied *ad libitum*. Semen samples were collected from the rams using an artificial vagina, during the breeding season (between January and March, 2013), once a week. Semen samples with following criteria were pooled and used in the experiments. Volume (≥ 0.75 – 2.00 mL), sperm concentration (≥ 3×10^9 per mL), mass movement (≥ 4), total motility (≥ 80.00%) and morphology abnormalities (< 10.00%).

Semen processing and parameters. Tris–egg yolk extender [Tris 3.03 g, fructose 1.25 g, citric acid 1.70 g, penicillin (100,000 IU), streptomycin (100 μg) in 100 mL of distilled water and egg yolk 20% (v/v)] was used for semen dilution.[20] Each pooled semen sample divided into five equal aliquots and each aliquot was diluted with egg yolk-Tris extender containing various concentrations of E2 (100 μmol) or α-ZOL (100 pmol, 100 nmol and 100 μmol) and/or vehicle at a final concentration of 200×10^6 sperm per mL. The diluted semen samples were stored at 4 ˚C and were evaluated after different times of incubation (0, 24, 48, 72 and 96 hr). In each time point, the semen samples placed in 35 ˚C water bath for 15 min and then were evaluated for the sperm viability, motility and membrane integrity.

Sperm motility. The motility of sperms were analyzed using computer-assisted sperm motility analysis (CASA) system (Sperm 2.1; VideoTest, St. Petersburg, Russia). Forty μL of each sperm sample was added to 1000 μL Tris-based extender (3.03 g Tris, 1.25 g fructose, and 1.70 g citric acid in 100 mL of distilled water) and after 15 min, 10 μL of sperm sample was placed on a previously heated slide (37 ˚C) and covered with a cover slide (22 × 22 mm). At least ten non-consecutive and randomly selected microscopic fields per sample were assessed for motility parameters using

a phase contrast microscope (Labomed LX400, Labo America Inc., Fremont, USA) that was equipped with a heat stage. The motility parameters that were measured included, curvilinear velocity (VCL; μm sec^{-1}), straight-line velocity (VSL; μm sec^{-1}), average path velocity (VAP; μm sec^{-1}), amplitude of lateral head displacement (ALH; μm sec^{-1}), and straightness of path (STR; %) which was calculated as VSL/VAP × 100.

After determination of sperm motility by CASA, sperms with VCL ≥ 40μm sec^{-1} were counted as motile sperm, and sperms with VCL ≥ 60 μm sec^{-1} and STR ≥ 60% were counted as of sperms with progressive motility.

Analysis of sperm membrane integrity. Sperm membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) based on previously described method.[21] Fifty μL diluted sperm sample was added to 500 μL distilled water and 1000 μL hypo-osmotic solution (fructose 1.35 g, sodium citrate 0.73 g and 0.10 g BSA dissolved in 100 mL distilled water) in microtubes and incubated for 1 hr at 37 ˚C. Then, 10 μL of mixture was placed on a slide and covered with a cover slide. Three hundred spermatozoa were examined from at least 10 fields of different parts of the slide under 400× magnification, using a phase-contrast microscope. The percentage of sperms with swollen and curled tails was determined.

Assessing the level of total nitrite/nitrate. The total nitric oxide (NO) content of the stored semen samples following the supplementation with various concentrations of α-ZOL and 100 μmol E2 at different time points was measured according to the Griess reaction.[22] In Griess reaction, nitric oxide rapidly converted into the stable nitrite, which in an acidic environment, nitrite is converted to HNO_2. In reaction with sulphanilamide, HNO_2 forms a diazonium salt, which reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form an azo dye that can be detected at 540 nm wavelength. The NO content of the examined semen samples was expressed as nmol per mL.

Measurement of reducing capacity. The total antioxidant capacity of the stored semen samples was measured by previously described method.[23] Briefly, at low pH, which was achieved by addition of acetate buffer (300 mM, pH 3.6), reduction of Fe^{III}–TPTZ complex to the ferrous form, produced an intensive blue color that could be measured at 593 nm. Aqueous solution of $FeSO_4.7H_2O$ and appropriate concentrations of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.

Statistical analysis. The results were represented as mean ± SEM. Differences between treatments and/or storage times were examined by two-way ANOVA, followed by Dunnett's test in SAS software (SAS Institute Inc. version 9.4, Cary, USA). A *p*-value less than 0.05 was considered significant.

Results

To examine the sperm plasma membrane integrity, HOST was conducted and the results showed that *α*-ZOL induced remarkable damage to the plasma membrane integrity during the liquid storage. Moreover, the greatest amount of damage to the plasma membrane was found at the highest concentration of *α*-ZOL (100 μmol) during the storage time. α-ZOL reduced the sperm plasma membrane integrity at 100 μmol concentration after 96 hr incubation (Table 1).

Effects of E2 and *α*-ZOL supplementation on sperm motility and sperm progressive motility are shown in Tables 2 and 3, respectively. The total motility of sperm significantly decreased at higher concentration of *α*-ZOL (100 nmol and 100 μmol) after 48 to 96 hr. Also, the progressive motility decreased after 72 to 96 hr at 100 μmol of *α*-ZOL and after 72 hr at 100 nmol.

Sperm motility parameters assessment by CASA showed that sperm supplementation with α-ZOL was able to significantly decrease the rate of sperm motility parameters (Table 4). The most significant detrimental effect of α-ZOL was found on VCL parameter at 100 μmol concentration of *α*-ZOL immediately and after 48 to 96 hr incubation and at 100 nmol concentration immediately and after 72 hr incubation time. VAP was affected immediately and after 48 to 96 hr storage only at 100 μmol concentration *α*-ZOL. Significant decrease in VSL was observed only at high concentration of *α*-ZOL after 48 and 96 hr incubation in comparison with non-treated samples ($p < 0.05$). Significant decrease in sperm ALH parameter was recorded at 100 nmol concentration of

Table 1. Effect of different concentrations of E2 and *α*-ZOL on sperm plasma membrane integrity (%) in pooled semen diluted in Tris–egg yolk extender and stored for various time periods at 4 ˚C.

Treatment	Storage time (hr)				
	Fresh/diluted	24	48	72	96
Control	84.80 ± 1.20^{a}	78.80 ± 1.71^{b}	73.40 ± 1.56^{c}	68.40 ± 2.11^{d}	62.20 ± 1.68^{e}
***α*-ZOL (100pM)**	85.00 ± 1.30^{a}	76.60 ± 1.63 ^{b}	71.60 ±1.43^{c}	67.00 ± 2.33^{d}	61.60 ± 2.31^{e}
***α*-ZOL (100nM)**	84.60 ± 1.16^{a}	74.80 ± 1.77^{b*}	70.00 ± 1.51^{c*}	65.20 ± 2.47^{d}	60.60 ± 1.77^{e}
***α*-ZOL (100μM)**	83.60 ± 1.50^{a}	73.0 ± 2.40^{b*}	66.40 ±1.86^{c*}	61.20 ± 1.98^{d*}	55.40 ± 1.46^{e*}
E2 (100μM)	83.60 ± 1.69^{a}	77.60 ± 1.28^{b}	70.40 ± 1.63^{c*}	68.80 ± 1.82^{d}	64.40 ± 2.33^{e}

Different superscripts indicate significant differences between data in each raw ($p < 0.05$).
* Asterisk indicates significant differences between the E2 and *α*-ZOL supplemented and non-supplemented groups ($p < 0.05$).

α-ZOL immediately and after 72 hr the supplementation and at 100 µmol concentration of α-ZOL after 48 to 96 hr incubation time ($p < 0.05$). Significant reduction in sperm STR parameter was found only in diluent containing α-ZOL in comparison to control group after 48 and 72 hr incubation ($p < 0.05$).

Table 2. Effects of different concentrations of E2 and α-ZOL on the percentage of sperm motility in pooled semen diluted in Tris–egg yolk extender and stored for various time periods at 4 ˚C.

Treatment	Storage time (hr)				
	Fresh/diluted	24	48	72	96
Control	86.40 ± 0.69^{a}	84.80 ± 0.64^{a}	80.30 ± 1.33^{b}	76.60 ± 0.87^{c}	67.20 ± 3.07^{d}
α-ZOL (100pM)	87.60 ± 1.04^{a}	82.60 ± 0.85^{b}	77.30 ± 1.05^{c}	74.60 ± 1.55^{d}	64.90 ± 1.76^{e}
α-ZOL (100nM)	86.60 ± 0.84^{a}	83.30 ± 1.36^{a}	78.40 ± 1.58^{b}	72.00 ± 2.12^{c}	64.70 ± 2.69^{d}
α-ZOL (100µM)	87.30 ± 0.77^{a}	81.40± 1.74^{b}	74.60 ± 1.75^{c*}	69.40 ± 1.52^{d*}	60.60 ± 1.94^{e*}
E2 (100µM)	86.90 ± 0.85^{a}	83.70 ± 0.95^{a}	80.60 ± 1.18^{b}	72.10 ± 0.81^{c}	67.30 ± 1.88^{d}

Different superscripts indicate significant differences between data in each raw ($p < 0.05$).
* Asterisk indicates significant differences between the E2 and α-ZOL supplemented and non-supplemented groups ($p < 0.05$).

Table 3. Effects of different concentrations of E2 and α-ZOL on the percentage of sperm with progressive motility in pooled semen diluted in Tris–egg yolk extender and stored for various time periods at 4 ˚C.

Treatment	Storage time (hr)				
	Fresh/diluted	24	48	72	96
Control	68.90 ± 1.66^{a}	69.00 ± 0.77^{a}	61.80 ± 0.62^{b}	58.80 ± 1.01^{c}	49.90 ± 3.09^{d}
α-ZOL (100pM)	71.40 ± 0.79^{a}	66.40 ± 1.10^{a}	61.40 ± 1.47^{b}	55.20 ± 1.60^{c}	47.10 ± 1.57^{d}
α-ZOL (100nM)	72.70 ± 0.57^{a}	65.60 ± 0.70^{b}	60.30 ± 2.03^{c}	54.20 ± 0.89^{d*}	45.20 ± 2.49^{e}
α-ZOL (100µM)	70.50 ± 1.00^{a}	64.30 ± 1.37^{b}	57.00 ± 0.86^{c}	49.30 ± 0.91^{d*}	40.00 ± 1.02^{e*}
E2 (100µM)	73.00 ± 1.14^{a}	65.10 ± 1.37^{a}	60.40 ± 1.35^{b}	55.20 ± 1.04^{c}	47.50 ± 2.45^{d}

Different superscripts indicate significant differences between data in each raw ($p < 0.05$).
* Asterisk indicates significant differences between the E2 and α-ZOL supplemented and non-supplemented groups ($p < 0.05$).

Table 4. Effect of the E2 and α-ZOL supplementation on sperm motility parameters in pooled semen diluted in Tris–egg yolk extender and stored for various time periods at 4 ˚C.

Parameter	Treatment	Storage time (hr)				
		Fresh/diluted	24	48	72	96
VAP (µm sec^{-1})	**Control**	54.20 ± 1.34^{a}	51.40 ± 1.87^{a}	44.40 ± 2.41^{b}	38.40 ± 1.51^{c}	32.70 ± 2.19^{d}
	α-ZOL(100 pM)	54.30 ± 1.81^{a}	48.80 ± 1.38^{b}	43.40 ± 2.05^{c}	35.90 ± 0.99^{d}	31.70 ± 1.79^{e}
	α-ZOL(100 nM)	54.90 ± 2.27^{a}	48.60 ± 2.17^{b}	41.00 ± 1.19^{c}	35.90 ± 0.99^{d}	31.20 ± 0.69^{e}
	α-ZOL(100 µM)	50.30 ± 1.00^{a*}	47.80 ± 1.90^{a}	38.30 ± 1.29^{b*}	33.90 ± 0.94^{c*}	26.60 ± 0.78^{d*}
	E2(100 µM)	57.50 ± 2.15^{a}	47.00 ± 1.04^{b}	42.30 ± 1.59^{c}	36.10 ± 1.81^{d}	31.80 ± 1.06^{e}
VCL (µm sec^{-1})	**Control**	111.60 ± 2.42^{a}	105.00 ± 1.99^{b}	99.40 ± 2.29^{c}	93.60 ± 1.85^{d}	82.40 ± 1.81^{e}
	α-ZOL(100 pM)	108.00 ±1.94^{a}	103.10 ± 2.69^{b}	96.00 ± 2.22^{c}	89.80 ± 1.09^{d}	79.90 ± 1.40^{e}
	α-ZOL(100 nM)	107.30 ± 1.52^{a*}	103.70 ± 2.12^{a}	95.40 ± 2.16^{b}	86.10 ± 0.87^{c}	78.20 ± 0.90^{d}
	α-ZOL(100 µM)	107.40 ± 2.07^{a*}	101.20 ± 3.43^{b}	89.80 ± 2.30^{c*}	81.80 ± 0.84^{d*}	71.30 ± 0.65^{e*}
	E2(100 µM)	114.00 ± 1.77^{a}	105.70 ± 1.77^{b}	99.10 ± 1.82^{c}	90.90 ± 1.17^{d}	81.80 ± 1.87^{e}
VSL (µm sec^{-1})	**Control**	47.90 ± 1.41^{a}	45.50 ± 1.64^{a}	38.90 ± 0.02^{b}	33.40 ± 1.45^{c}	28.70 ± 2.32^{d}
	α-ZOL(100 pM)	48.00 ± 1.65^{a}	42.80 ± 1.33^{b}	37.90 ± 1.93^{c}	31.30 ± 1.92^{d}	27.50 ± 1.90^{e}
	α-ZOL(100 nM)	49.30 ± 1.96^{a}	42.50 ± 1.38^{b}	35.60 ± 1.09^{c}	31.30 ± 0.94^{d}	27.20 ± 0.68^{e}
	α-ZOL(100 µM)	44.50 ± 0.65^{a}	41.40 ± 1.60^{a}	33.50 ± 1.35^{b*}	29.60 ± 0.89^{c}	23.00 ± 0.84^{d*}
	E2(100 µM)	50.60 ± 2.09^{a}	40.80 ± 1.04^{b}	36.80 ± 1.44^{c}	31.70 ± 1.35^{d}	27.70 ± 1.08^{e}
ALH (µm)	**Control**	1.76 ± 0.06^{a}	1.66 ± 0.04^{a}	1.60 ± 0.06^{b}	1.55 ± 0.03^{c}	1.35 ± 0.03^{d}
	α-ZOL(100 pM)	1.65 ± 0.06^{a}	1.61 ± 0.07^{a}	1.52 ± 0.06^{b}	1.50 ± 0.03^{c}	134.00 ± 0.04^{d}
	α-ZOL(100 nM)	1.62 ± 0.08^{a*}	1.60 ± 0.07^{a}	1.54 ± 0.07^{a}	1.39 ± 0.05^{b*}	1.31 ± 0.03^{c}
	α-ZOL(100 µM)	1.65 ± 0.07^{a}	1.58 ± 0.07^{a}	1.46 ± 0.06^{b*}	1.35 ± 0.03^{c*}	1.19 ± 0.02^{d*}
	E2(100 µM)	1.81 ± 0.05^{a}	1.71 ± 0.05^{a}	1.63 ± 0.07^{b}	1.52 ± 0.04^{c}	1.38 ± 0.05^{d}
STR (%)	**Control**	82.70 ± 1.01^{a}	81.10 ± 0.66^{a}	77.60 ± 0.52^{b}	76.00 ± 1.47^{c}	69.70 ± 1.54^{d}
	α-ZOL(100 pM)	82.90 ± 0.31^{a}	79.90 ± 0.58^{b}	75.60 ± 0.82^{c}	73.70 ± 1.44^{d}	69.00 ± 0.92^{e}
	α-ZOL(100 nM)	86.00 ± 0.29^{a}	79.80 ± 0.09^{b}	76.20 ± 1.14^{c}	73.20 ± 0.89^{d}	68.20 ± 0.98^{e}
	α-ZOL(100 µM)	82.90 ± 0.71^{a}	78.20 ± 0.98^{b}	74.00 ± 0.89^{c*}	71.70 ± 0.55^{d*}	66.20 ± 1.14^{e}
	E2(100 µM)	82.30 ± 0.95^{a}	78.70 ± 0.96^{a}	75.90 ± 0.99^{b}	73.30 ± 0.78^{c}	69.60 ± 1.32^{d}

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; ALH: amplitude of lateral head displacement; and STR: straightness.
Different superscripts indicate significant differences between data in each raw ($p < 0.05$).
* Asterisk indicates significant differences between the E2 and α-ZOL supplemented and non-supplemented groups ($p < 0.05$).

The TAC value at the time of semen dilution (0 hr) was only increased at 100 µmol level of α-ZOL, while lower concentrations resulted in an elevation of TAC when the cells were incubated for long time. Maximum antioxidant power was obtained 48 hr after incubation at 100 nmol of α-ZOL concentration, while maximum antioxidant capacity was observed after 96 hr of incubation, only at 100 pmol. Significant decrease in TAC value was found at 100 µmol concentration of E2 in comparison to non-treated sample after 24 hr incubation (Fig. 1).

Assessment of the total nitrite/nitrate level during the storage periods of the ram sperms in extender solution containing different concentrations of α-ZOL and at 100 µmol concentration of E2, is shown in Figure 2. We failed to show any significant changes in the nitrite/ nitrate levels during storage time with different concentrations of α-ZOL and at 100 µmol concentration of E2 in comparison to the control group.

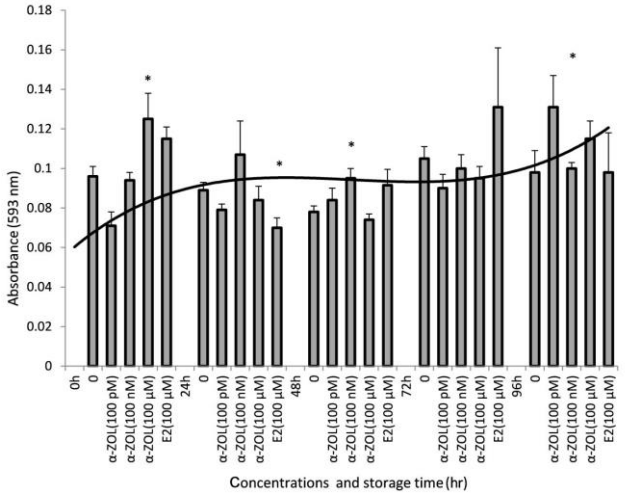

Fig. 1. Effects of different concentrations of E2 and *α*-ZOL on TAC in ram semen storage for various time periods at 4 ˚C.

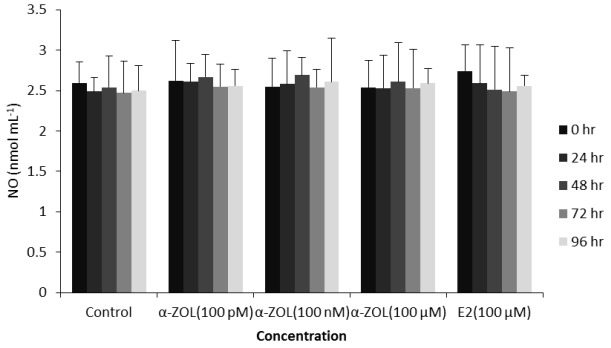

Fig. 2. Effects of different concentrations of E2 and *α*-ZOL on total NO level in ram semen storage for various time periods at 4 ˚C.

Discussion

In the present study, we analyzed the effects of mycotoxin *α*-ZOL and E2 on sperms viability, motility, plasma membrane integrity, nitric oxide (NO) levels and total antioxidant capacity during the liquid storage of semen under *in vitro* condition. 17 β-estradiol (E2) was used as a positive control for 'estrogen-like' effects. In general, estrogens are considered as female hormones that involved in fertility and reproduction. However, in recent years it has been shown that estrogens play an important role in males, too.[24-26] 17 β-estradiol is the strongest natural estrogen and until recent years it has been shown that the biological effects of E2 on cells take place through genomic pathways and via estrogen receptors alpha and beta (ERα, ERβ).[27] Luconi and co-workers[28] in a study on human sperm, reported the existence of active estrogen superficial receptors with 29 KDa molecular weight, which E2 binding to these receptors results in an increase of intracellular calcium concentration in sperms. This theory that steroid hormones on sperm function may act through pathways other than the classical genomic routes, has attracted particular attention for mature sperms since the latter are transcriptionally inactive.[29]

Currently analyzing different parameters of sperm (motility and morphology) is the most favorable approach for the evaluation of reproductive ability. Spermatozoa for movement toward oocytes need to have normal mobility characters and sperm motility is one of the most important features in the evaluation of fertility potential. In a study it has shown that human and animal oocytes fertility rate has a direct correlation with sperm motility, which provides necessary conditions for traverse the sperm in reproductive tract and penetration through the zona pellucida of the oocytes and fusion within the plasma membrane.[30] Furthermore a number of studies have indicated that the quantitative assessment of sperm motility by CASA reflects the fertilizing capacity of human spermatozoa. Among the other sperm movement parameters, VCL has been determined as the most significant and independent CASA parameter, which highly correlates with the rate of fertilization in the general male population.[31-34]

The results of the present study indicate that under *in vitro* conditions, *α*-ZOL was able to decrease most of the motility parameters, in a concentration-and time-dependent manner. The decrease in parameters related to VCL, VSL and VAP were observed in our study. The most significant detrimental effect of *α*-ZOL was found on VCL and VAP parameters at 100 µmol concentration during 96 hr of storage at 4 ˚C. At the same concentration, the changes in parameters related to motility patterns of sperm (ALH and STR) were observed during the longer incubation times in liquid and chilled medium. While, the semen supplementation with E2 at 100 µmol concentration did not influence sperm parameters during incubation times. Therefore, the decrease of velocity of sperm movement, might be because of mycotoxin *α*-ZOL which had detrimental effects on fertility of ram sperms.

Previous studies showed the toxic effects of zearalenone and its derivatives via competitively binding to specific estrogen receptors and by modification of steroid metabolism.[35-38]

In the present study, when sperms were treated with various concentrations of α-ZOL, maximum antioxidant capacity was observed immediately after semen supplementation at concentration 100 μmol of α-ZOL. When the sperms were incubated for the longer time, the antioxidant capacity were decreased gradually and touched the lowest value at 48 hr after incubation. Antioxidant capacity was increased probably due to the activation of antioxidant enzymes (catalase, superoxide dismutase and peroxidase Glutathione). Maximum antioxidant capacity was obtained 48 and 96 hr after incubation with α-ZOL at concentration of 100 nmol and 100 pmol, respectively. Another reason for the negative effect of α-ZOL on ram sperm parameters was likely because of its effect on antioxidant capacity. Oxidizing agents (reactive oxygen species, ROS or oxygen free radicals) are considered as destructive factors affecting the sperm quality in the male reproductive system.[39] Antioxidant enzymes in the sperm cells are not able to withstand ROS excessive, therefore, protect plasma membrane that cover the sperm acrosome and tail.[40] The negative impact of excessive ROS production or low antioxidant capacity on sperm motility may be explained by two facts that either reduced sperm motility is a reflection of ROS-related reduction of phosphorylation of axoneme protein or due to limitation of enzymes' activities (G-6-PDH), which in turn result in reduction of glutathione in the cells.[41-42] In addition, once spermatozoa were dead, they release enzymes that exert toxic agents causing changes in kinematic parameters such as reduction of live sperm motility.[43]

The results of the current study indicated that under *in vitro* condition, α-ZOL affected the sperm plasma membrane integrity after 24 of incubation. According to Ball *et al*. decrease in sperm viability could be due to the effects of free radicals on membrane structure.[44] In intact and normal membrane, unsaturated fatty acids are important in the creation of sperms psychometric and fluidity proportions in vaginal environment. Whereas, only live sperms with normal plasma membrane integrity able to penetrate through the zona pellucida of the oocytes and subsequent fusion with the plasma membrane.[45-46]

Therefore, according to the results obtained under *in vitro* conditions, α-ZOL negative effects on the sperms motility and viability parameters might be related to its effect on plasma membrane integrity and consequently on the sperm movement toward oocytes. Thus, it could be concluded that α- ZOL may affect the fertilization ability of ram sperm. Our results confirm, the previously described negative effects of ZEA and α-ZOL on boar sperm viability and motility parameters.[16]

In conclusion, this study demonstrated the negative effects of α-ZOL supplementation of semen on ram sperms motility and plasma membrane integrity, time- and dose-dependently in liquid storage. Moreover, the negative effects of α-ZOL could be related to the induction of oxidative stress that is generated at the highest concentration. Therefore, existence of α-ZOL mycoestrogen in animal feed may be counted as a great concern in the animal husbandry.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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