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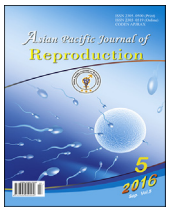

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Comparative blood and seminal plasma oxidant/antioxidant status of Arab stallions with different ages and their relation to semen quality

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ABSTRACT

Objective: To investigate the antioxidant/oxidant levels in blood serum and seminal plasma of Arab stallion with different ages and their relation to semen quality.
Methods: Healthy Arabian stallions ($n = 57$), were divided into three groups. Young (5–10years), Moderate (11–16 years) and Old stallions (>16 years) were subjected to semen evaluation. Seminal plasma and blood samples were collected and stored at −20 °C for measuring glutathione reduced, nitric oxide, Malondialdehyde, ascorbic acid, copper and zinc.
Results: Old stallions had significantly greater ($P < 0.05$) ejaculate volume, % live sperm, and total sperm number compared to young and moderate aged groups. The moderate age horses had significantly the lowest ($P < 0.05$) sperm concentration. Compared to young horses, serum zinc concentrations of moderate and old horses were significantly high ($P < 0.0\,001$), but NO concentrations were significantly ($P < 0.05$) low. Seminal plasma zinc, ascorbic acid and nitric oxide concentrations were significantly ($P < 0.05$ and 0.01) high in young stallion group. No significant correlations were observed between seminal zinc, copper, MDA and semen variables. Meanwhile, significant negative correlations were observed between seminal plasma ascorbic acid concentration and all semen variables except total sperm number and sperm abnormities %. Significant correlations were observed between reduced glutathione and both of sperm motility and % of live sperm. Nitric oxide concentrations correlated directly with individual sperm motility but adversely with total sperm number.
Conclusion: Stallion age has significant effect on some semen variables, antioxidant/oxidant status of either blood serum or seminal plasma.

1. Introduction

The sperm cell is one of the aerobic organisms that require oxygen. Spermatozoa are rich in targets for oxidative attack and the major source of ROS within the sperm is mitochondria [1]. Depleting Adenosine tri-phosphate within mitochondria causes loss of sperm motility, viability and capacity for fertilization [2,3]. Defective or immature sperms and semen leukocytes produces more ROS and causes sperm dysfunction in men [2] and stallions [4,5]. Physiological low levels of ROS are required for the capacitation process in bulls [6], and normal sperm function in fertile men [2]. Hydrogen peroxide is responsible for the acrosome reaction [7]. Sperm DNA damage of infertile men resulted from high levels of sperm-derived ROS [4,8,9]. The presence of little cytoplasm within the head of spermatozoa makes them deficient in antioxidants and DNA-repair systems [2]. In stallion semen, ROS damage cells by changing lipids, proteins and DNA. It was found that peroxidative stress triggers the mitogen activated protein kinase cascade and the extracellular signal-regulated protein kinase phosphorylation [5]. Spermatozoa are potentially susceptible to peroxidative damage caused by excess ROS due to high amounts of polyunsaturated fatty acids in membrane phospholipids and to sparse cytoplasm. High levels of ROS might be detrimental to stallion sperm survival during storage [10]. Spermatozoa plasma membrane contained high polyunsaturated fatty acids [11], so the deleterious effects of lipid

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peroxidation on spermatozoa are loss of motility and fertilizing capacity [12]. The epididymis and seminal plasma possesses a complex antioxidant system aiming to prevent oxidative damages on sperm lipids, proteins and DNA [13]. The main enzymatic scavenger in the stallion cauda epididymal fluid and seminal plasma is catalase [14]. Although equine spermatozoa have limited GPx and SOD-like activity but equine seminal plasma contains high activity of SOD [15]. Fertile stallion's seminal plasma contains lower concentration of ascorbic acid than infertile ones. From ascorbic acid antioxidant properties to diminish lipid peroxidation [15,16], to regenerate other antioxidant molecules, such as B-carotene, vitamin E and reduced glutathione from their relevant radical species [18]and top reserves membrane integrity of cooled equine sperm [19]. During equine semen storage partial removal of seminal plasma is required to improve sperm survival [20]. Partial seminal plasma removal during semen processing preservation results in removal of oxidative stress scavengers and in turn subjecting spermatozoa to the damaging effect of excessive reactive oxygen species [21].

Higher copper levels in blood serum [22], seminal plasma and sperm are conjugated with infertility [23], poor sperm maturation, motility and fertility in boar [24], ram and bull [25], water buffaloes [26]. In stallions, seminal plasma zinc deficiency not only affects testes development and spermatogenesis [22,27] but also plays a role in protecting sperms during freezing [28].

This study aimed to compare blood serum and seminal plasma copper, zinc, reduced glutathione, ascorbic acid and nitric oxide in Arab stallion with different ages and their relationship to sperm quality.

2. Materials and methods

2.1. Semen collection, evaluation and semen analysis

A total of 57 healthy Arabian stallions, between 6 and 26 aged years were used during this study. These stallions belonged to Police Academy Stud (Cairo), and several private horse studs (Giza) Egypt, and they have been used as sires in the regular natural breeding program of the stud. Stallions were divided according to age into young (5–10) years old; moderate (11–15) years old; and old (>15) years old. Three semen ejaculates were collected for each stallion at weekly interval. At the time of collection, early in the morning, a mare in estrus was used as a mount animal. Semen was collected using a lubricated and pre-warmed (45–50 °C) Colorado model artificial vagina with an inline filter to separate the gel fraction. Immediately after collection, semen samples were transferred to a well-equipped laboratory and the gel-free portion of the ejaculate was evaluated for volume, progressive motility, and concentration was determined by conventional methods [29]. Spermatozoa motility was examined and recorded using a pre-warmed stage of microscope (200×). Sperm concentration was determined with a hemocytometer. Total sperm count per ejaculate was calculated from volume and sperm concentration. The pH was determined with test strips (Merck, Darmstadt, Germany) and the morphological examination of semen samples was determined using nigrosin–eosin stain [30]. To obtain seminal plasma, an aliquot of semen was immediately centrifuged after semen collection at 1 000 ×g for 10 min. The procedure was repeated with the supernatant of the first centrifugation to insure sperm free seminal plasma and stored at −20 °C until they were analyzed.

2.2. Blood sampling and analytical methods

Blood for was collected from the external jugular vein into vacuum tubes. Immediately after collection, the blood samples were centrifuged at 1 000 ×g for 15 min, and sera was stored at −20 °C until they were analyzed.

2.3. Blood antioxidant and biochemical analysis

Serum and seminal plasma glutathione (GSH) reduced [31], nitric oxide (NO) [32], lipid peroxide product (Malondialdehyde, MDA) [33], Ascorbic acid [34], copper and zinc [35] were measured using commercial kits (Bio Diagnostic, Egypt).

2.4. Statistical analysis

All data were analyzed using the SPSS statistical software [36]. The data were expressed as mean ± standard error of means (SEM). Independent sample *t*-test, simple one way ANOVA and Pearson correlation test were used. Duncan Multiple Range test was used to differentiate between significant means at $P < 0.05$.

3. Results

Seminal plasma (Table 1) contained significantly high concentrations of zinc ($P < 0.0001$), NO ($P < 0.05$), reduced glutathione ($P < 0.0001$), ascorbic acid ($P < 0.001$) and MDA ($P < 0.05$) but nearly similar copper compared to their serum levels.

Semen characteristics of the three age groups (Table 2) showed a significant ($P < 0.05$) increase of ejaculate volume and % of live sperms ($P \leq 0.05$), but tendency to a decrease of abnormal sperm % for horses older than 15 y (O). Stallions of moderate age had significantly low sperm cell concentrations ($P < 0.05$) and total sperm count ($P < 0.01$) compared to young and old stallions (Table 2).

Blood serum of old stallions had significantly ($P < 0.05$) high zinc but significantly low NO ($P < 0.01$), compared to the other age groups (Table 3). Moderate aged stallions had significantly low zinc ($P = 0.03$), GHD and MDA ($P < 0.05$).

Seminal plasma of young stallions (Y, Table 4) had significantly the highest zinc ($P < 0.01$), NO ($P < 0.0001$) and ascorbic acid ($P < 0.01$) concentrations compared to the other age groups and tended to have high MDA and GHD ($P > 0.05$).

Seminal plasma zinc had only negative correlation with sperm individual motility ($r = -0.50$; $P < 0.05$) and tended to correlate with total sperm number ($r = 0.39$; $P > 0.05$), but copper had no significant correlations with semen evaluation parameters (Table 5). Ascorbic acid had significant negative correlation with sperm individual motility ($r = -0.69$; $P = 0.02$),

Table 1
Mean ± SEM of blood serum and seminal plasma antioxidant status, zinc and copper of Arab stallions.

Parameter	Serum	Semen	*P*-value
Zinc (mg/dL)	0.39 ± 0.09	2.04 ± 0.37	0.0001
Copper (mg/dL)	0.368 ± 0.05	0.457 ± 0.03	0.09
MDA (nmol/mL)	4.26 ± 0.45	6.92 ± 2.09	0.05
NO (μmol/L)	23.04 ± 1.46	30.56 ± 2.34	0.02
Reduced glutathione (mg/L)	9.83 ± 0.66	23.33 ± 6.61	0.0001
Ascorbic acid (mg/L)	19.76 ± 4.49	61.59 ± 16.54	0.001

Table 2
Mean ± SEM of semen parameters of young (5–10 years), moderate (11–15 years), and old (>15 years) Arab stallions.

Groups	Young	Moderate	Old	*P*-value
Age	5.03 ± 0.20[a]	13.25 ± .41[b]	17.67 ± .18[c]	0.0001
Semen volume (mL)	38.33 ± 8.53[a]	40.0 ± 10.37[a]	67.78 ± 6.016[b]	0.02
pH	6.92 ± 0.14	6.93 ± 0.12671	7.05 ± 0.099	0.67
Mass motility	3.33 ± 0.33[b]	2.60 ± .24[a]	3.11 ± .11[ab]	0.12
Individual sperm motility %	76.67 ± 3.33	68.00 ± 4.89	77.22 ± 1.69	0.11
Live sperm %	76.33 ± 3.9[ab]	70.33 ± 3.77[a]	80.88 ± 1.18[b]	0.05
Abnormal sperm (%)	15.25 ± 0.57[ab]	20.00 ± 3.53082[b]	11.67 ± 1.32[a]	0.07
Sperm cell concentration ($\times 10^6$/mL)	369.50 ± 34.44[b]	283.50 ± 30.43[a]	370.33 ± 14.68[b]	0.05
Total sperm count ($\times 10^9$)	21.66 ± 3.60[b]	12.25 ± 1.05[a]	24.64 ± 2.13[b]	0.002

Means with different superscripts (a, b, c) are significantly different at $P < 0.05$.

Table 3
Mean ± SEM of blood serum antioxidant status, zinc and copper of Arab stallions.

Groups	Young	Moderate	Old	*P*-value
Zinc (mg/L)	0.37 ± 0.01[a]	0.35 ± 0.04[a]	0.61 ± 0.07[b]	0.03
Copper (mg/dL)	0.31 ± 0.04[a]	0.55 ± 0.03[b]	0.48 ± 0.04[b]	0.07
Ascorbic acid (mg/L)	26.04 ± 8.09	14.99 ± 0.62	10.05 ± 2.49	0.6
Reduced glutathione (GHD; mg/dL)	10.59 ± 0.99	7.17 ± 0.04	8.54 ± 1.00	0.5
Nitric Oxide (NO)	24.32 ± 2.02[b]	12.38 ± 0.26[ab]	5.54 ± 0.71[a]	0.01
MDA (nmol/mL)	5.03 ± 0.59[b]	2.41 ± 0.12[a]	3.18 ± 1.41[ab]	0.05

Means with different superscripts (a, b) are significantly different at $P < 0.05$.

Table 4
Mean ± SEM of seminal plasma antioxidant status, zinc and copper of Arab stallions.

Groups	Young	Moderate	Old	*P*-value
Zinc (mg/L)	2.93 ± 0.75[b]	1.29 ± 0.26[a]	1.45 ± 0.30[a]	0.002
Copper (mg/dL)	0.33 ± 0.06[a]	0.50 ± 0.02[b]	0.50 ± 0.02[b]	0.002
Ascorbic acid (mg/L)	61.59 ± 16.54[b]	15.580 ± 2.86[a]	6.43 ± 2.67[a]	0.006
Reduced glutathione (GHD, mg/dL)	23.33 ± 6.61	7.13 ± 1.41	9.57 ± 1.65	0.069
Nitric oxide (NO)	31.99 ± 4.22[b]	7.71 ± 0.040[a]	8.29 ± 2.91[a]	0.0001
MDA (nmol/mL)	6.92 ± 2.01	3.15 ± 0.90	3.57 ± 0.98	0.09

Means with different superscripts (a, b) are significantly different at $P < 0.05$.

live sperm % ($r = -0.64$; $P = 0.035$) and sperm cell concentration ($r = -0.67$; $P = 0.02$), but has a positive correlation with abnormal sperm % ($r = 0.54$; $P > 0.05$). Reduced glutathione (GHD) has strong correlation with sperm individual motility ($r = 0.81$; $P = 0.005$), live sperm % ($r = 0.64$; $P = 0.03$), and sperm cell concentrations ($r = 0.58$; $P = 0.05$). NO had a significant positive correlation with sperm individual motility ($r = 0.69$; $P = 0.01$), and live sperm % ($r = 0.467$; $P = 0.05$), but negatively with total sperm number ($r = -0.69$; $P < 0.05$), and semen volume ($r = -0.68$; $P < 0.05$). MDA has positive correlation with total sperm number ($r = 0.64$; $P < 0.05$), semen volume ($r = 0.58$; $P = 0.05$), but a negative correlation with abnormal sperm % ($r = -0.74$; $P < 0.05$).

4. Discussion

Seminal plasma is an essential part of the ejaculate and it is involved in sperm functional activity, metabolism, survival and transport through the female reproductive tract. However, the deleterious action of equine seminal plasma on sperm motility is still controversial [37–39]. Moreover, a decrease of frozen-thawed semen motility was noticed when seminal plasma was not fully removed prior to cryopreservation [37]. Evaluating trace elements, antioxidant status and oxidative stress could be supportive to traditional semen evaluation in equine. In the current work, older stallions had significantly greater ejaculate volume, % live sperm, sperm concentration and total sperm number compared to the other two young groups, whereas the middle aged group showed lowest value for sperm concentration than younger and older groups. No significant age effect on mass and individual sperm motility, pH and total sperm abnormalities was observed. Similar to our results, age of Arab stallions had no effect on sperm motility and sperm concentration but affected total sperm abnormalities [40].

Table 5
Correlations among seminal plasma antioxidant, zinc and copper concentration and seminal variables.

	Volume	Individual motility	Live sperm %	Abnormal sperm %	Sperm cell concentration	Total sperm number
Age	0.29	−0.14	0.34	−0.39	0.29	0.41
Zinc	0.35	−0.50[b]	0.06	−0.24	−0.18	0.39
Copper	−0.29	−0.27	−0.24	0.27	0.07	−0.28
Ascorbate	−0.22	−0.69[b]	−0.64[b]	0.54	−0.67[b]	−0.25
GSH	−0.37	0.81[a]	0.64[b]	−0.06	0.58	−0.44
NO	−0.68[b]	0.69[b]	0.48	0.09	0.46	−0.69[b]
MDA	0.58	0.098	0.32	−0.74[b]	0.27	0.64[b]

GSH: Reduced glutathione; NO: Nitric oxide; MDA: Malondialdehyde.
[a] Correlation is significant at the 0.01 level. [b] Correlation is significant at the 0.05 level.

Similar to our findings, sperm abnormalities of aged Arab stallions were low compared to younger ones [40].

Zinc and copper in the body fluids are involved in cell protection against reactive oxygen species. Abnormal concentrations of seminal plasma zinc and copper are correlated with human infertility [41]. Copper exacerbate the damaging effects of ROS on sperm function [9]. The increase of copper concentration than normal values induces a toxic effect on spermatozoa by reducing oxidative processes and glycolysis that may reduce motility and viability [41]. Seminal plasma contained >6 folds higher zinc than blood serum and the improved semen parameters of old stallions not only refer to its higher level in blood serum but also in seminal plasma. Higher zinc concentrations were also reported in seminal plasma of stallions [28], and boar [24]. As well as, blood serum zinc and seminal plasma zinc significantly related, and no correlation between blood serum zinc, copper concentration and the stallion's semen quality were even reported [42]. Seminal plasma zinc concentration was not related with either sperm density or motility [43]. Although a non significant correlations between zinc and semen parameters except sperm individual motility were observed in stallions of this study but a significant positive correlation between zinc and semen volume and sperm concentration [44]. In rams, a significantly positive correlation between zinc and sperm motility percentage and sperm concentration [45].

Although seminal plasma copper levels of stallions were low [28], but significant individually variations in stallion seminal plasma copper concentration were also reported [28]. No correlation between copper levels of seminal plasma and semen variables in stallion [44]. However, serum copper was related to stallion semen characteristics [42]. Age-dependent differences in the concentration of zinc and copper within the blood serum were found [42]. Concentrations of zinc and copper in blood and semen of stallion may be related to the individual stallions, variation in dietary regimen and environmental condition, more than its relations to stallion age. In rams, significant negative correlation between copper, sperm motility %, sperm concentration and spermatozoa abnormalities [45].

Seminal plasma ascorbic acid had been conjugated to males with history of sub-fertility or idiopathic infertility [46]. Nevertheless, lower ascorbic acid concentrations were observed in normozoospermic than normal men [47]. Moreover, addition of ascorbic acid to extender was beneficial for maintaining membrane integrity of cooled equine sperm [19].

In the current study, seminal plasma and blood serum ascorbic acid concentrations were significantly higher in younger stallions and were decreasing with advancing age. In contrast higher ascorbic acid concentrations were observed in older stallions that increased with increasing age and adversely related to fertility [40]. In horses of the current study, seminal plasma content of ascorbic acid was 3 fold higher than serum levels, but in men seminal plasma concentration of ascorbic acid was 8–10-fold higher than blood [48]. Concentration of ascorbic acid in testes and seminal plasma is extremely sensitive to its blood levels [49].

GSH is a tri-peptide widely distributed inside living cells. It plays an important role in cell protection from the deleterious effect of oxidative stress, directly and as a cofactor of GSH peroxidase [50]. The inclusion of GSH in cryopreservation extender has had variable results in several species [51,52]. In the present study, concentrations of serum ascorbic acid, reduced glutathione and MDA and NO in seminal plasma were significantly high but age had no significant effect on circulating GHD and MDA and tended to affect its concentrations in seminal plasma. NO plays important roles in sperm physiology by its participation in the regulation of sperm motility and capacitation [53,54].

In absence of peroxidation inducers, only relatively little peroxidation was noted in fresh sperm cells whereas some mid-piece peroxidation was noted for frozen-thawed sperm cells that progressed to sperm head leading to loss of head membrane [55].

The exact role of NO in mammalian sperm physiology seems contradictory; low NO-levels are useful, while elevated NO levels seems harmful [56]. Spermatozoa are also capable of producing NO a production that is crucial for sperm motility, capacitation, and fertilization [57,58]. In stallion, the recorded positive correlation between NO production and post-thawing sperm motility and velocity, suggesting a crucial role for maintaining sperm function during cryopreservation [59]. NO has been shown to have detrimental effects on normal sperm functions inhibiting both motility and sperm competence for zona binding [60].

We concluded that the age of the stallion determinately affected semen variables, oxidant and antioxidant status of either blood or seminal plasma.

Conflict of interest statement

The authors declare that they have no competing interest.

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References

[1] Koppers AJ, De Luliis GN, Finnie JM, McLaughlin EA, Aiteken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008; **93**: 3199-3207.
[2] Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 1994; **16**: 259-267.
[3] Aitken RJ, Marshall GJ. Human spermatozoa: the future of sex. *Nature* 2002; **415**: 963.
[4] Ball BA, Vo AT, Baumber J. Generation of reactive oxygen species by equine spermatozoa. *Am J Vet Res* 2001; **62**(4): 508-515.
[5] Almog T, Naor Z. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. *Mol Cell Endocrinol* 2008; **282**: 39-44.
[6] O'Flaherty CM, Beorlegui NB, Beconi MT. Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. *Theriogenology* 1999; **52**: 289-301.
[7] Gordon I. Capacitating bovine sperm. In: *Laboratory production of cattle embryos*. 2nd ed. Gordon I, CABI Publishing, CAB International; 2003, p. 158-175.
[8] Sigman M, Zini A. Semen analysis and sperm function assays: what do they mean? *Semin Reprod Med* 2009; **27**: 115-123.
[9] Aitken RJ, De Luliis GN, Finnie JM, Hedges A, McLachlan R. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 2010; **25**(10): 2415-2426.

[10] Aurich C. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. *Anim Reprod Sci* 2005; **89**: 65-75.
[11] Brouwers JF, Silva PFN, Gadella BM. New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. *Theriogenology* 2005; **63**: 458-469.
[12] da Silva CM, Macias-Garcia B, Miro-Moran A, Gonzalez-Fernandez L, Morillo-Rodriguez A, Ortega-Ferrusola C, et al. Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. *J Pineal Res* 2011; **51**: 172-179.
[13] Vernet P, Aitken RJ, Drevet JR. Antioxidant strategies in the epididymis. *Mol Cell Endocrinol* 2004; **216**: 31-39.
[14] Ball BA, Gravance CG, Medina V, Baumber J, Liu IK. Catalase activity in equine semen. *Am J Vet Res* 2000; **61**: 1026-1030.
[15] Baumber J, Ball BA. Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. *Am J Vet Res* 2005; **66**: 1415-1419.
[16] Knight JA, Blaylock RC, Searles DA. The effects of vitamins C and E on lipid peroxidation in stored erythrocytes. *Ann Clin Lab Sci* 1993; **23**: 51-56.
[18] Halliwell B. Vitamin C: antioxidant or pro-oxidant in vivo. *Free Radic Res* 1996; **25**: 439-454.
[19] Aurich JE, Schönherr U, Hoppe H, Aurich C. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. *Theriogenology* 1997; **48**: 185-192.
[20] Morrell JM, Georgakas A, Lundeheim N, Nash D, Davies Morel MCG, Johannisson A. Effect of heterologous and homologous seminal plasma on stallion sperm quality. *Theriogenology* 2014; **82**: 176-183.
[21] Ball BA. Oxidative stress, osmotic stress and apoptosis: impact on sperm function and preservation in the horse. *Anim Reprod Sci* 2008; **107**: 257-267.
[22] Telisman S, Cvitkovic P, Jurasovic J, Pizent A, Gavella M, Rocic B. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environ Health Perspect* 2000; **108**: 45-53.
[23] Aydemir B, Kiziler AR, Onaran, Alici B, Ozkara H, Akyolcu MC. Impact of Cu and Fe concentrations on oxidative damage in male infertility. *Biol Trace Elem Res* 2006; **112**: 193-203.
[24] Massanyi P, Trandzik J, Nad P, Korenekova B, Skalicka M, Toman R, et al. Concentration of copper, iron, zinc, cadmium, lead, and nickel in boar semen and relation to the spermatozoa quality. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2003; **38**: 2643-2651.
[25] Massanyi P, Trandzik J, Nad P, Korenekova B, Skalicka M, Toman R, et al. Concentration of copper, iron, zinc, cadmium, lead, and nickel in bull and ram semen and relation to the occurrence of pathologic spermatozoa. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2004; **39**: 3005-3014.
[26] Tabassomi M, Alavi-Shoushtari SM. Effects of *in vitro* copper sulphate supplementation on the ejaculated sperm characteristics in water buffaloes (Bubalusbubalis). *Veterinary Res Forum* 2013; **4**: 31-36.
[27] Cigankova V, Mesaros P, Bires J, Ledecky V, Ciganek J, Tomajkova E. The morphological structure of the testis in stallions with zinc deficiency. *Slovak Vet J* 1998; **23**: 97-100.
[28] Barrier-Battut, Dellajarraud H, Legrand E, Bruyas J-F, Fiéni F, Tainturier D, et al. Calcium, magnesium, copper, and zinc in seminal plasma of fertile stallions, and their relationship with semen freezability. *Theriogenology* 2002; **58**: 229-232.
[29] Kenney RM, Hurtgen J, Pierson R, Witherspoon D, Simons J. Theriogenology and the equine, Part II, the stallion semen examination. *J Soc Theriogenology* 1983; **9**: 3-54.
[30] Dowsett KF, Osborne HG, Pattie WA. Morphological characteristics of stallion spermatozoa. *Theriogenology* 1984; **22**: 463-472.
[31] Beutler F, Duron O, Kelly MB. Improved methods for the determination of blood glutathione. *J Lab Clin Med* 1963; **61**: 882-890.
[32] Montgomery HAC, Dymock JF. The determination of nitrite in water. *Analyst* 1961; **86**: 414-416.
[33] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 1979; **95**: 351-358.
[34] Harris IJ, Mapson LW, Wang YL. Vitamin method: a simple potentiometric method for determining ascorbic acid, suitable for use with coloured extracts. *Biochem J* 1942; **36**(1–2): 183-195.
[35] Ventura S, King EJ. Determination of copper and zinc in blood serum. *Biochem J* 1951; **48**(5): lxi-lxii.
[36] *SPSS: statistical package for social science, PC software, Version16*. 2007.
[37] Nishikawa Y. Motility and fertilizing ability of frozen horse spermatozoa. In: *Proceedings of the 7th international symposium on zootechnology*; 1972, p. 155-167 [cited by Amann RP, Cristanelli MJ, Squares EL. Proteins in stallion seminal plasma. *J Reprod Fertil Suppl* 1987; **35**:113-120].
[38] Pickett BW, Sullivan JJ, Byers WW, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. *Fertil Steril* 1975; **26**: 167-174.
[39] Braun J, Torres-Boggino F, Hochi S, Oguri N. Effect of seminal plasma on motion characteristics of epididymal and ejaculated stallion spermatozoa during storage at 5°C. *Dtsch Tierärztl Wochenschr* 1994; **101**: 301-304.
[40] Waheed MM, El-Bahr SM, Al-haider AK. Influence of seminal plasma antioxidants and osteopontin on fertility of the Arabian horse. *J Equine Vet Sci* 2013; **3**: 705-709.
[41] Cevk M, Tuncer PB, Tafidemr U, Ozurtafi T. Comparison of spermatological characteristics and biochemical seminal plasma parameters of normozoospermic and oligoasthenzoospermic bulls of two breeds. *Turk J Vet Anim Sci* 2007; **31**: 381-387.
[42] Danek J, Wisniewski E, Krumrych W. The dependence between concentrations of zinc, copper, and calcium in blood serum and the characteristics of stallion semen. *Med Weter* 1999; **55**(4): 259-264.
[43] Abou-Shakra FR, Ward NI, Everard DM. The role of trace element in male infertility. *Fertil Steril* 1989; **52**: 307-310.
[44] Pesch S, Bergmann M, Bostedt H. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. *Theriogenology* 2006; **66**: 307-313.
[45] Mahsud T, Jamil H, Qureshi ZI, Asi MN, Lodhi LA, Waqas MS, et al. Semen quality parameters and selected bio-chemical constituents level in plasma of *Lohi* rams. *Small Ruminant Res* 2013; **113**: 175-178.
[46] Colagar AH, Marzony ET. Ascorbic Acid in human seminal plasma: determination and its relationship to sperm quality. *J Clin Biochem Nutr* 2009; **45**: 144-149.
[47] Thiele JJ, Friesleben HJ, Fuchs J, Ochsendorf FR. Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. *Hum Reprod* 1995; **10**(1): 110-115.
[48] Jacob RA, Pianalto FS, Agee RE. Cellular ascorbate depletion in healthy men. *J Nutr* 1992; **122**: 1111-1118.
[49] Chinoy NJ, Mehta RR, Seethalakshmi L, Sharma JD, Chinoy MR. Effects of vitamin C deficiency on physiology of male reproductive organs of Guinea pigs. *Int J Fertil* 1986; **31**: 232-239.
[50] Atmaca G. Antioxidant effects of sulfur-containing amino acids. *Yonsei Med J* 2004; **45**(5): 776-788.
[51] Anel-López L, Alvarez-Rodríguez M, García-Álvarez O, Alvarez M, Maroto-Morales A, Anel L, et al. Reduced glutathione and Trolox (vitamin E) as extender supplements in cryopreservation of red deer epididymal spermatozoa. *Anim Reprod Sci* 2012; **135**(1–4): 37-46.
[52] Câmara DR, Silva SV, Almeida FC, Nunes JF, Guerra MM. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. *Theriogenology* 2011; **76**(2): 342-350.
[53] Hellstrom WJ, Bell M, Wang R, Sikka SC. Effect of sodium nitroprusside on sperm motility viability and lipid peroxidation. *Fertil Steril* 1994; **61**: 1117-1122.

[54] Lewis SEM, Donnelly ET, Sterling ESL, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial for sperm motility. *Mol Hum Reprod* 1996; **11**: 873-878.

[55] Neild DM, Brouwers JF, Colenbrander B, Agüero A, Gadella BM. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. *Mol Reprod Dev* 2005; **72**(2): 230-238.

[56] Vignini A, Nanetti L, Buldreghini E, Moroni C, Ricciardo-Lamonina G, Mantero F, et al. The production of peroxynitrite by human spermatozoa may affect sperm motility through the formation of protein nitrotyrosine. *Fertil Steril* 2006; **85**: 947-953.

[57] Francavilla F, Santucci R, Macerola B, Ruvolo G, Romano R. Nitric oxide synthase inhibition in human sperm affects sperm-oocyte fusion but not zona pellucida binding. *Biol Reprod* 2000; **63**: 425-429.

[58] Goud PT, Goud AP, Diamond MP, Gonik MP, Abu Soud H. Nitric oxide extends the oocyte temporal window for optimal fertilization. *Free Radic Biol Med* 2008; **45**: 453-459.

[59] Ferrusola CO, González Fernández L, GaGarcý BM, Salazar-Sandoval C, Rodrýguez AM, Martinez HR, et al. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. *Biol Reprod* 2009; **81**: 1106-1111.

[60] Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 2008; **59**: 2-11.