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Influence of mineral supplementation on oxidative stress, ovarian follicles growth and reproductive hormone concentration in cyclic Arab mares

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ABSTRACT

Objective: To study the effect of minerals supplementation in improving brood mare antioxidant status, ovarian activity and some reproductive hormone profiles. **Methods:** Five Arab broodmares were supplemented with multi-minerals in their drinking water for three months (Treated) and five control mares received the same ration with no mineral (control) belonging to Police Academy horse stud. All mares were subjected to ovarian ultrasonography and blood sampling at weekly intervals during mineral supplementation. Leptin, estradiol, progesterone and insulin like growth factor -1 were assayed with radioimmunoassay. Mineral concentrations in blood serum were determined using atomic absorption spectrometer. Nitric oxide, lysozymes, total antioxidant capacity and lipid peroxide were also measured. **Results:** Diameter of the first large follicle (Dominant) and the second large follicle (Subordinate) was higher in treated mares during both estrous and early luteal phases. Moreover diameter of corpus luteum was higher in treated mares compared to control. Mineral supplementation decreased significantly (P=0.004) levels of leptin but insignificantly improved insulin like growth factor -1 in treated mares. Levels of progesterone, estradiol were insignificantly lower in treated mares. There was a significant increase in levels of lysozymes (P=0.0001) and total antioxidants (P<0.006) but a significant decrease in lipid peroxide (P<0.0001) in treated mares. Nitric oxide did not change greatly by mineral supplementation. Zinc (P=0.004), copper (P=0.009), Iron (P=0.001), cobalt (P=0.004) and magnesium (P=0.03) levels were significantly higher in treated mares. **Conclusions:** Mineral supplementation improved the animals' oxidant antioxidant balance, reproductive hormones and ovarian cyclicity and their supplementation is recommended before any breeding programs.

1. Introduction

Micronutrients play a central role in metabolism and in the maintenance of tissue function. The most important category of these micronutrients is the trace elements. Zinc[1,2], Copper [3,4] and Iron [5, 6] are a dietary trace minerals that, in addition to their many essential functions in growth and developments, are essential for the function of the immune system cells [1]. Magnesium also plays a fundamental role as a cofactor in more than 300 enzymatic reactions involving energy metabolism and nucleic acid synthesis [7].

Several mineral supplements have been extensively studied in animals in either inorganic or organic forms. In horses, zinc-L-selenomethionine was more effective than $NaSeO_3$ at increasing plasma Selenium [8] Selenium yeast was also more effective than Na selenite in increasing total Selenium in blood, mainly as consequence of a greater increase of the proportion of selenium comprised as selenium methionine but it did not modify GPX-1 activity in mature horses [9]. Some studies found no difference in utilization of minerals from organic and inorganic sources [10]. Others found that Cu-Lysine seemed to be better absorbed than $CuSO_4$ and absorption of Zn-Methionine and $ZnSO_4$ were not different, these results are tempered by the observation of abnormally high fecal and urinary excretion values for Cu and Zn [11]. Exercise in horses has an effect on some trace minerals. The iron levels were not changed by exercise or treatment alone but increased when the horses had been supplemented and exercised. The copper level and the copper/zinc ration increased as a result of exercise in both

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treated and untreated horses. Supplementation with vitamin E and selenium had an important effect on the serum concentrations of calcium, potassium, copper, iron, and the copper/zinc ratio [12]. In mares, maternal diet during the last one-third of gestation affects placental efficiency and colostral IgG [13], maternal plane of nutrition and selenium supplementation affected mare and foal plasma, muscle, and colostrum selenium concentrations, but not Gsh-Px activity [14]. To our knowledge, no data was available concerning the effect of multi-mineral supplementation on cyclic mares, estrous reproductive hormones and conception. So this study was designed to evaluate ovarian, steroid hormones and some immune parameters (antioxidants) responses in Arab mares supplemented with commercial inorganic multi-mineral in addition to leptin and insulin like growth factor-1.

2. Materials and methods

2.1. Animals and experimental design

Ten Arab brood mares belonging to Police Academy (Abaseia horse farm) of average 350-400 kg body weight were selected for performing this experiment. Mares were 5-14 years old. Mares were sequentially monitored throughout 4 months for evaluation of their reproductive status by ultrasonography. Mares were kept in open yards during the day and in stalls during night.

Treated mares (n=5) were supplemented with a mineral mixture (TS-biove Laboratories, Aques, France) at a dose level of 15 mL/100 kg body weight in drinking water each other day for 12 weeks. Each liter of supplemented minerals consisted of magnesium hydroxide (30.0 g), calcium hydroxide(25.0 g), zinc oxide (3.26 g), citric acid(6.0 g), sodium chloride(16.5 g), copper chloride (3.0 g), manganese chloride(8.7 g), cobalt chloride(0.1 g), iron chloride solution(15.0 mL) and phosphoric acid diluted (27.0 mL). Five other brood mares received no mineral supplement and served as control

2.2. Ultrasonographic examination

A NOVEK scanner (Germany) equipped with an endo-rectal linear array B-mode real time multi-frequency 2.6- 7.5 MHz transducer belonging to Police Academy was used to evaluate both ovaries and uterus. Number of non echogenic (black) follicles in the same scan with largest follicle was counted and the diameter of the largest two was recorded. A homogenous echogenic (grey) corpus luteum diameter was recorded the week after recording the ovulating follicle. Estrus was detected by observing animal behaviour and detection of follicle >3 cm in diameter [15].

2.3. Blood samples

Blood samples were collected via Jugular venipuncture at weekly intervals the same day of conducting ultrasound examination. Sera were harvested and stored at -20 ℃ until hormonal assay and antioxidant analysis.

2.4. Mineral analysis

Different mineral concentrations in each sample were determined by means of atomic absorption spectrometer.

2.5. Hormone assaying

Insulin like growth factor-1 [16] (IGF-1; BioSource Europe S. A. Belgium) Daughaday & Rotwein 1989 and Mutli-Species Leptin [17] (Linco Research, St. Charles, MO) commercial Kit were estimated by radioimmunoassay (RIA). The limit of sensitivity, intra- and inter-assay coefficients of variation were 3.4 ng/mL, 1.9% and 4.1% and were 1.0 ng/mL, 2.8% and 8% respectively for IGF-1 and leptin. Estradiol (E_2) was assayed using RIA commercial kit of Biosource laboratories [18]. Intra- and inter-assay coefficients of variation were 5.9 and 10.1%. Sensitivity of the assays was 2.0 pg /mL. Progesterone (P_4) was determined by RIA procedure using commercial kits supplied by Diagnostic Laboratories, USA [19]. Intra- and inter-assay coefficients of variation were 4.9 and 6.1%. Sensitivity of the assay was 0.02 ng/mL

2.6. Immunity and antioxidants measurements

Lysozyme activity [20] was measured by the lysoplate assay method. Briefly, heat-killed Micrococcus lysodeikticus (Sigma, 500 mg/L) were suspended in agarose gel (1%). Melt agarose was poured in Petri dishes to a depth of 4 mm. Twelve wells 1.5 mm in diameter were cut in the agarose. Wells were filled with 2 μL with standard dilutions of chicken egg white lysozyme (0-500 μg/mL) or with serum samples. Petri dishes were incubated at room temperature (23 to 25 ℃) for at least 18 h. Diameters of lysis zones were determined directly with a dial caliper (Mitutoya; obtained from Laboratory Supplies Co.,Hicksville,N.Y.) accurate to 0.02 mm.The results for standards were plotted on a semilogarithmic graph and the values for samples were extrapolated from this reference curve.

For measuring serum nitrite [21] 100 μL of serum samples were mixed with an equal volume of freshly prepared Greiss reagent, incubated for 10 min at room temperature and absorbency measured at 570 nm using a micro titer plate reader. The nitrite level in serum samples was calculated by comparing the optical density against the nitrite standard curve of sodium nitrite in distilled water.

Antioxidant concentrations [22] and lipid peroxide [23] were measured using colorimetric diagnostic kits (Bio-Diagnostic, Egypt).

2.7. Statistical analysis

Descriptive statistics for studied variables were performed using the SPSS for Windows [24]. The effect of estrous phase on ovarian parameters and leptin hormone was analyzed using split simple one-way ANOVA. The Duncan's Multiple Range test was used in separating differences between significant means. Student t-Test was used to study the effect of treatmenton hormones, mineral and antioxidants. Pearson bivariate correlation coefficient was also processed.

3. Results

A non significant increase in number of total follicles during the late luteal phase was observed in mineral supplementation mares (Table 1) compared to cntrol.

Table 1

The effect of mineral supplementation on leptin levels and some ovarian parameters in mares during different phases of estrous cycle.

Parameters	Estrous phase		Early luteal phase		Late luteal phase	
	Control	Treatment	Control	Treatment	Control	Treatment
Leptin (ng/mL)	1.93±0.58	1.31±0.59	1.57±0.45	1.08±0.18	0.60±0.60	0.62±0.19
Follicles of left ovary	2.41±0.32	2.22±0.46	3.00±.045	2.22±0.40	2.40±0.67	3.80±0.97
Follicles on right ovary	1.91±0.37	1.67±0.49*	2.59±0.42	2.13±0.35*	3.00±0.32	3.67±1.20*
Total follicles number	3.65±0.35	3.33±0.65	5.16±.0.65	4.11±0.63	5.40±0.93	6.00±2.00
Dominant follicle diameter/cm	3.24±0.18	3.49±0.13**	2.06±.0.90	2.19±0.29 **	2.61±0.22	2.45±0.11**
Subordinate follicle diameter/cm	2.63±0.15	2.71±0.25**	1.59±0.08	1.85±0.29**	2.08±0.20	1.73±0.24**
Corpus luteum diameter/cm	–	–	2.06±0.12	2.33±0.14	3.00±0.24	3.49±0.49

*P<0.05, compared with control, ** P<0.01 compared with control.

Diameter of the dominant and subordinate follicles was significantly high in treated mares during the estrous and early luteal phases but control mares had large dominant and subordinate follicle diameter during late luteal phase. Moreover, there is a non significant increase in the diameter of the corpus luteum in treated mares during early and late luteal phase.

Disrespecting the stage of the estrous phase, leptin concentration declined significantly after trace mineral supplementation (Table 2) but no significant variation is robserved in leptin levels either before or after mineral supplementation within stages of estrous cycle (Table 1). Levels of leptin declined during both estrus and early luteal phase after mineral supplementation but no change is clears during the late luteal phase.

There is a non significant increase in serum levels of Insulin like growth factor-1 in treated mares and a slight decrease in both estradiol and progesterone (Table 2) after mineral supplementation. Leptin levels are significantly low in treated mares.

Table 2

Effect of mineral supplementation on progesterone, estradiol, insulin like growth factor -1 and leptin in mares

Traits	Control	Treated	P-value
Progesterone(ng/mL)	8.73±2.84	6.85±1.15	0.2
Estradiol (pg/mL)	7.87±2.34	7.03±3.54	0.8
IGF(ng/mL)	234.42±17.54	322.95±13.97	0.4
Leptin (ng/mL)	2.48±0.32	1.23±0.20	0.004

Lysozyme levels increased significantly (P=0.0001) in mineral supplemented mares. There is no significant difference in nitric oxide (Table 3) between treated and control mares. There is a significant increase (P=0.006) in antioxidants concentrations accompanied with a significant (P=0.0001) decrease in the lipid peroxide significantly in supplemented mares (Table 3). All concentrations of supplemented minerals are significantly higher in mineral supplemented mares (Table 4).

Copper has a negative non significant correlation with estradiol (r = – 0.28), progesterone (r= – 0.17) and leptin (r= – 0.499) and a positive high non significant correlation with IGF-1(r= 0.88). Zinc has negative significant correlation with progesterone (r= – 0.90, P=0.01) and leptin (r= – 0.69; P=0.05) but has a positive non significant correlation with IGF-1 (r = 0.92; P=0.077) and estradiol (r=0.34). A positive high non significant correlation was found between leptin with both progesterone (r =0.86, P=0.066) and IGF-1(r =0.99, P=0.08).

Table 3

Effect of mineral supplementation on lysozyme activity, nitric oxide levels, antioxidant activity and lipid peroxidation.

Oxidative stress markers	control	Treated	P-value
Lysozyme activity(μ g/mL)	127.67±11.65	187.37±5.48	0.0001
Nitric oxide (μ moL)	11.31±0.27	11.81±0.43	0.206
Total Antioxidants (mM /L)	0.33±0.02	0.49±0.09	0.006
Lipid peroxide (nmol/mL)	11.03±0.75	6.96±0.41	0.0001

Table 4

levels of some minerals before and after supplementation.

Minerals	Control	Treated	P -value
Magnesium(mg/dL)	2.37±0.28	3.31±0.09	0.03
Zinc(μ g/dL)	82.25±6.07	112.48±3.06	0.004
Copper(μ g/dL)	79.00±5.93	107.28±4.55	0.009
Iron(μ g/dL)	83.75±3.90	132.50±7.03	0.001
Cobalt(μ g/dL)	0.41±0.04	1.61±0.26	0.004
Selenium (μ g/dL)	62.28±5.87	64.70±2.24	0.7

4. Discussion

Horses in governmental farms are fed mainly barely grains in addition to Egyptian clover (Trifolium alexandrinum) during winter so any minerals and vitamins deficient in barely grains are compensated by that present in the green clover. During summer while most mares are late pregnant this green clover is replaced by another available green corn stems which are not as the same quality as Egyptian clover so animals may suffer from mineral deficiency for several months during periods of great demands where animals withdraw most of their reserves from minerals and vitamins also the summer heat stress decreases the amount of nutrient intake by each animal. Parturitions occur usually during late autumn and winter where no sufficient reserves are available to the animals which may reflect on estrous regularity and next foal heat conception. Cows receiving organic trace minerals exhibited higher pregnancy rates to AI than those receiving inorganic trace minerals [25]. This improved reproductive performance reported in dairy cows receiving organic mineral supplements was referred to the improved repair of damaged uterine tissue following calving

[26]. Trace mineral supplementation also improved pregnancy rate to AI compared with cows not supplemented with Cu, Zn, or Mn for more than 1 yr[27].

The increase in number of total follicles in mineral supplemented mares during late luteal stage is due to the significant increase in the number of follicles on both ovaries. Although diameter of dominant, subordinate follicles and corpus luteum is high in treated mares but this increase in the diameter was accompanied by a non significant decrease in the levels of estradiol and progesterone. The increase in their diameter may refer to the increase in insulin like growth factor-I hormone.

eptin is mainly an adipocyte-secreted protein that is involved in the regulation of food intake and energy balance [28]. Leptin is secreted into the periphery in proportion to body fat percentage in horses [29] and also responds to changes in energy balance in Thoroughbred mares [30]. Leptin secretion increases during summer than winter [31]. This seasonal variation in circulating leptin has been reported to exist only in mature mares [32]. The increased levels of leptin during the estrous phase may be attributed to its effect in inducing hyperemia and leakage in the follicle which facilitates extrusion of the follicular content leading to ovulation [33]. In contrast to the decrease in leptin levels during all estrous phases in treated mares of our study, an increase in leptin levels near foal heat ovulation was recorded in Arab mares [15] this may refer to the difference in management and ration formulation and in turn body condition between the two farms. However, in seasonal breeding mares, perturbations in leptin secretion observed in some high BCS mares are not associated with alterations in ovarian activity or the estrous cycle. A 35% of mares with high body condition scores (BCS) displayed estrous cycles or had considerable follicular activity during the winter exhibited a persistent hyperleptinemia [34, 35]. Increased concentrations of this hormone in circulation might be associated with the restart or maintenance of ovarian cyclicity in Lusitano mares [36]. Leptin has been found to be not only associated with reproduction but also with immune response [37]. Contrary to our results, serum leptin was found to be positively associated with serum copper (r=0.197, P=0.02) and the serum zinc/copper ratio (r=-0.182, P=0.03) in healthy human. These results suggest that copper and not zinc has an effect on serum leptin levels [38]. Similar to our results, both plasma leptin and zinc increased with no-training elite female judo athletes and plasma zinc correlated positively with plasma leptin (r=0.83; P=0.002) in the no-training condition [39]. Contrary to mare results, plasma copper did not change and correlated positively with plasma leptin (r=0.66; P=0.05) after training [39]. However, in young male judo athletes, plasma zinc was associated with leptin (r=0.82; P<0.05) while copper was associated with plasma leptin in females (r=0.66; P<0.05) [40]. Plasma zinc, copper, and energy homeostasis may be involved in regulation of plasma leptin [40]. Also maternal serum leptin levels were positively correlated with maternal zinc levels [41]. In women with thyroid disorder, a weak correlation (r=0.28, P=0.032) was found between leptin and the Zn/Cu ratio [42]. The correlation between leptin and Zn, and the Zn/Cu ratio was negative (r=-0.359, P<0.05; r=-0.361, P<0.05, respectively) in pooled subjects which is similar to that recorded in mares. When subjects were divided based on the presence or absence of hypertension, there was a negative correlation between leptin and Zn (r=-0.375, P<0.05) as well as leptin and Zn/Cu ratio (r=-0.398, P<0.05) in non hypertensed subjects [43]. This controversy in results may refer to difference in human and horses, age, exercise, sex and physiological condition.

Growth factors, in particular the insulin-like growth factor (IGF) system, are thought to play a key role in ovarian follicular growth and atresia [44]. The IGFs have a variety of effects on follicular and luteal cells, including stimulation of steroidogenesis, via increased availability of steroid precursors and up-regulation of steroidogenic enzyme expression and activity. Intrafollicular IGF-I may be involved in selection of dominant follicles in mares [3, 45]. In mineral supplemented mares, IGF-1 increased in serum and mares were mature and our means fall within the range recorded in other study [46] where older mature mares have lower insulin-like growth factor-I concentrations (295 *vs* 369 *ng*/mL) when compared with young growing mares [46]. This increase in IGF-1 levels in multi-mineral supplemented mares may refer to copper, since rats fed low and marginal copper had lower serum IGF-I than those fed high dietary copper [47]. Also, in both plasma and follicular fluids and that intrafollicular IGF bioavailability must exceed a threshold level before ovulation can occur [48]. Plasma concentrations of IGF-I also decrease with exercise and decreasing level of nutrition of horses [49]. The increase in IGF-1 levels is also referred to zinc supplemented in mineral mixture since zinc-induced rise in serum IGF-I was partly due to increased feed intake in weaned piglets [50].

The increase in the total antioxidants concentration and the decrease in the lipid peroxide levels in treated mares indicated improve animals' oxidant and antioxidants balance and in turn improved immunity. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds. ROS can initiate lipid peroxidation and cause cellular damage to tissues. Immune cells are particularly sensitive to oxidative stress because their membranes contain high concentrations of polyunsaturated fatty acids that are very susceptible to peroxidation, and they produce large amounts of ROS when stimulated [3, 4]. The increase in total antioxidants capacity and the decrease in lipid peroxide in broodmares of this study are similar to that found in trained thoroughbred horses, since an antioxidant imbalance was observed after three months in the control group compared the antioxidant supplement group with significant sex- or age-related differences. Moreover, trained thoroughbred horses undergo significant changes of several blood antioxidant markers and that oral antioxidant supplementation might partially counterbalance these changes by improving the hydrophilic, lipophilic and enzymatic antioxidant blood capacity [51].

A number of trace minerals are required for functioning

of enzymes involved in the antioxidant defense system. Certain trace minerals may also affect immune cells via certain mechanisms distinct from antioxidant activities and any deficiency of these nutrients may depress immunity [52]. In agreement with Stabel *et al.* [53] deficiencies of specific nutrients can reduce immune responses and increase disease susceptibility. For example Copper deficiency results in decreased humoral and cell-mediated immunity. Zn deficiency impedes host defense systems, leading to increased susceptibility to a variety of pathogens and a deficiency and they added that iron regulates the function of T lymphocytes any deficiency in iron results in impaired cell-mediated immunity [1, 2]. Moreover, Manganese shares in regulating of immune response of the body. The decline in oxidative stress after multi mineral supplementation in mares of this study is attributed to improve in levels of trace elements supplemented including Zn, Cu, Mg, and iron. Many workers [45-56] reported that these elements reduce the free radicals and protect the cells from its harmful effect by their antioxidant action. In horses, exercise-induced changes of its oxidant/antioxidant balance and antioxidant supplementation is frequently recommended but blood oxidant/ antioxidant status of those horses is also influenced by breed, gender and age [57].

There was an increase in serum levels of Zn, Cu and Mg in brood mares of the present work. They are incorporated in superoxide dismuase and their increase reflects oxidant/ antioxidants balance and that mares could overcome disturbances in this balance during their breeding after mineral supplementation. Values of these minerals are higher than their values in growing horses [58]. Copper and zinc concentrations of the horses infected with Equine Herpesvirus-1 were lower than the control group ($P<0.001$), whereas iron concentration and the copper/zinc ratio of the infected group were higher than the control group ($P<0.05$ and $P<0.001$). The cobalt concentration was not found to be statistically different between two groups [59]. The horses with piroplasmosis had lower plasma levels of zinc and elevated copper. Consequently, the copper/zinc ratio was also higher than in the healthy controls [60].

Equine lysozyme [61] is a small basic protein which can hydrolyze the peptidoglycan layer in the cell walls of sensitive bacteria. In addition, lysozyme is capable of promoting bacterial aggregation and loss of viability in dependent of cell lysis. Lysozymes protect the host against pathogenic infection [62]. Equine lysozymes [EL) have been implicated in innate immunity which acts as a bacteriolytic enzyme identified in the many body fluids and tissues [63] such as saliva [64], cervical mucus [65], human tear[66], uterine secretions [67], milk and blood serum [68]. The increase in lysozyme activity in mineral supplemented mares is related to improved immuonological status of mares. After the start of mineral mixture addition to mares, the phagocyte activity evaluated by lysozyme amount significantly increase which may be attributed to the protecting effect of minerals on body cells specially immune cells from the oxidative stress or through direct participation between minerals and immunological activities pathway. In mares, temporary increase in lysozyme activity in uterine secretions was observed for 12 to 24 hours after bacterial challenge to overcome uterine bacterial inoculation [67]. Although values of lysozymes in saliva did not differ between immunodeficient and healthy subjects and also both age and sex has no significant effect on its values [68] but this study demonstrated a significant increase in lysozyme activity in treated mares which is related to improved health and reproduction? Also, Yue *et al.* [69] added that copper, zinc, manganese and iron increase activate of natural killer (NK) cell function. this hypothesis supported by Lastra *et al.* [70] who mentioned that, there was a significant increment in the peritoneal macrophages phagocyte index (macrophages ingested opsonized erythrocyte) and he suggested that the immunological role of zinc is most likely mediated by monocyte activation and cytokine release.

In conclusion, this study recommends using mineral supplementation before starting breeding since mineral supplementation improved animals' health by improving their immunological status and improved levels of some reproductive steroid hormones during estruos cycle and improved also normal ovarian follicular growth and corpus luteum development.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Weber G, Konieczyński P. Speciation of Mg, Mn and Zn in extracts of medicinal plants. *Anal Bioanal Chem* 2003; 375:1067-1073.

[2] Spears JWP, Weiss WP. Role of antioxidants and trace elements in health and immunity of transition dairy cows. *Vet J* 2008; 176: 70-76.

[3] Spears JW. Micronutrients and immune function in cattle. *Proceedings of the Nutrition Society* 2000; 59: 587-594.

[4] Weiss WP, Spears JW. Vitamin and trace mineral effects on immune function of ruminants. In: Sejrsen K, Hvelplund T, Nielsen MO. (eds.), Ruminant physiology.Utrecht: Wageningen Academic Publishers; *Utrecht. The Netherlands* 2006,p. 473-496.

[5] El Hendy HA, Yousef MI, AboEl-Naga NL Effect of dietary zinc deficiency on hematological and biochemical parameters and concentrations of zinc, copper, and iron in growing rats. *Toxicology* 2001; 167: 163-170.

[6] Sordillo LM. Factors affecting mammary gland immunity and mastitis susceptibility. *Livestock Prod Sci* 2005; 98: 89-99.

[7] Wong WY, Flik G, Groenen PM, Swinkels DW, Thomas CM,

Copius-Peereboom JH, *et al*. The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. *Reprod Toxicology* 2001; 15:131–136.

[8] Richardson SM, Siciliano PD, Engle TE, Larson CK, Ward TL. Effect of selenium supplementation and source on the selenium status of horses. *J Anim Sci* 2006; 84:1742–1748.

[9] Calamari L, Ferrari A, Bertin G. Effect of selenium source and dose on selenium status of mature horses. *J Anim Sci* 2009; 87:167–178.

[10]Baker LA, Wrigley MR, Pipken JL, Haliburton JT, Bachman RC. Digestibility and retention of inorganic and organic sources of copper and zinc in mature horses. In: *19th Equine Sci. Symp., Tucon, AZ. Equine Sci. Soc., Champaign, IL*. 2005,p. 162–165.

[11]Wagner EL, Potter GD, Gibbs PG, Eller EM, Scott BD, Vogelsang MM, *et al*. Copper and zinc balance in exercising horses fed 2 forms of mineral supplements. *J Anim Sci* 2011; 89 : 722–728.

[12]Yur F, Dede S, Deger Y, Kilicalp D. Effects of vitamin E and selenium on serum trace and major elements in horses. *Biol Trace Elem Res* 2008 ; 125: 223–228.

[13]Thorson JF, Karren BJ, Bauer ML, Cavinder CA, Coverdale JA, Hammer CJ. Effect of selenium supplementation and plane of nutrition on mares and their foals: foaling data. *Anim Sci* 2010; 88 :982–990.

[14]Karren BJ, Thorson JF, Cavinder CA, Hammer CJ, Coverdale JA. Effect of selenium supplementation and plane of nutrition on mares and their foals: selenium concentrations and glutathione peroxidase. *J Anim Sci* 2010; 88: 991–997.

[15]Abo El-Maaty AM, Gabr FI. Relation beween leptin and estradiol levels in Egyptian lactating Arab mares during foaling heat. *Anim Reprod Sci* 2010 ; 117: 95–98.

[16]Daughaday WH, Rotwein P. Insulin like growth factor I and II . Peptide, Messenger ribonucleic acid and gene structure. Serum and tissue concentrations. *Endocrine review* 1989; 10: 68–91.

[17]Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, *et al*. Leptin levels in human and rodent: Measurement of plasma leptin in ob RNA in obese and weight-reduced subjects. *Nature Med* 1995; 1 :1155–1161

[18]Metha RR. Subcellular concentrations of estrone, estradiol, androstenodione and 17- β hydroxysteroid dehydrogenase (17BOH-SOH) activity in malignant and nonmalignant human breast tissues. *Int J Cancer* 1987; 40: 305–308.

[19]Brutis CA, Ashwood ER. Tietz textbook of clinical chemistry. 2nd ed. Philadelphia:W.B. Saunders Company; 1994, p.1863.

[20]Jenzano JW, Hogan SL, Lundblad RL. Factors influencing measurement of human salivary lysozyme and turbidimetric assays. *J Clin Microbiol* 1986; 24: 963–967.

[21]Rajaraman V, Nonnecke BJ, Hammell DC, Horst RL. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. *J Dairy Sci* 1998; 81: 3278–3285.

[22]Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol* 2001; 54: 356–361

[23]Ohkawa H, Ohishi W, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351–358.

[24]SPSS (Statistical Package for Social Science) Computer software. Version 16. Inc, Chicago, IL, USA, 2007.

[25]Stanton TL, Whittier JC, Geary TW, Kimberling CV, Johnson AB. Effets of trace mineral supplementation on cow-calf performance, reproduction and immune function. *Prof Anim Sci* 2000; 16:121–127.

[26]Manspeaker JE, MG Robl, GH Edwards, LW Douglass. Chelated minerals: Their role in bovine fertility. *Vet Med* 1987;82:951–956.

[27]Ahola JK, Baker DS, Burns PD, Mortimer RG, Enns RM, Whittier JC, *et al*. Effect of copper, zinc, and manganese supplementation and source on reproduction, mineral status, and performance in grazing beef cattle over a two-year period. *J Anim Sci* 2004; 82: 2375– 2383.

[28]Zhang F, Basinski M, Beals JM, Briggs SL, Churgay LM, Clawson DK, *et al*, Crystal structure of the obese protein leptin E-100. *Nature* 1997; 387: 206–220.

[29]Kearns CF, McKeever KH, Roegner V, Brady SM, Malinowski K. Adiponectin and leptin are related to fat mass in horses. *Vet J* 2006; 172: 460–465.

[30]McManus C, Fitzgerald B. Effects of a single day of feed restriction on changes in serum leptin, gonadotropins, prolactin, and metabolites in aged and young mares. *Dom Anim Endocrinol* 2000; 19: 1–13

[31]Buff PR, Messer IV NT, Cogswell AM, Johnson PJ, Keisler DH, Ganjam VK. Seasonal and pulsatile dynamics of thyrotropin and leptin in mares maintained under a constant energy balance. *Dom Anim Endocrinol* 2007; 33: 430–436.

[32]Fitzgerald BP, McManus CJ. Photoperiodic versus metabolic signals as determinants of seasonal anestrus in the mare. *Biol Reprod* 2000; 63: 335–340

[33]Barkan D, Hurgin V, Dekel N, Amsterdam A, Rubinstein M. Leptin induces ovulation in GnRH-deficient mice. *FASEB J* 2005; 19: 133–135.

[34]Waller CA, Thompson DL Jr, Cartmill JA, Storer WA, Huff NK. Reproduction in high body condition mares with high versus low leptin concentrations. *Theriogenology* 2006; 66: 923–928.

[35]Romagnoli U, Macchi E, Romano G, Motta M, Accornero P, Baratta M. Leptin concentration in plasma and in milk during the interpartum period in the mare. *Anim Reprod Sci* 2007; 97: 180–185.

[36]Ferreira-Dias G, Claudino F, Carvalho H, Agrícola R, Alpoim-Moreira J, Robalo SJ. Seasonal reproduction in the mare: possible role of plasma leptin, body weight and immune status. *Domest Anim Endocrinol* 2005; 29: 203–213.

[37]Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cells immune response and reverses starvation induced immuno-suppression. *Nature* 1998: 394, 89–97

[38]Ohusi S, Al-Awadhi A, Abraham M, Abiaka C, George S. Serum copper levels and not zinc are positively associated with serum leptin concentrations in the healthy adult population. *Biol Trace Elem Res* 2003; 91:137–144

[39]Koury JC, de Oliveira Kde J, Lopes GC, de Oliveira AV Jr, Portella ES, de Moura EG, *et al*. Plasma zinc, copper, leptin, and body composition are associated in elite female judo athletes. *Biol Trace Elem Res* 2007 ; 115: 23–30.

[40]Casimiro-Lopes G, de Oliveira-Junior AV, Portella ES, Lisboa PC, Donangelo CM, de Moura EG, *et al*. Plasma leptin, plasma zinc, and plasma copper are associated in elite female and male judo athletes. *Biol Trace Elem Res* 2009 ;127: 109–115.

[41]Ozdemir U, Gulturk S, Aker A, Guvenal T, Imir G, Erselcan T. Correlation between birth weight, leptin, zinc and copper levels in maternal and cord blood. *J Physiol Biochem* 2007 ; 63:121–128.

[42]Chen MD, Song YM, Tsou CT, Lin WH, Sheu WH. Leptin concentration and the Zn/Cu ratio in plasma in women with thyroid disorder. *Biol Trace Elem Res* 2000;75: 99–105.

[43]Canatan H, Bakan I, Akbulut M, Halifeoglu I, Cikim G, Baydas G, *et al*. Relationship among levels of leptin and zinc, copper, and zinc/copper ratio in plasma of patients with essential hypertension and healthy normotensive subjects. *Biol Trace Elem Res* 2004; 100:117–123.

[44]Armstrong DG, Gutierrez CG, Baxter G, Glazyrin AL, Mann GE, Woad KJ, *et al*. Expression of mRNA encoding IGF–I, IGF–II and type 1 IGF receptor in bovine ovarian follicles. *J Endocrinol* 2000; 165: 101–113.

[45]Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR. Mechanism of follicle deviation in monovular farm species. *Anim Reprod Sci* 2003; 78: 239–257.

[46]Malinowski K, Christensen RA, Hafs HD, Scanes CG. Age and breed differences in thyroid hormones, insulinlike growth factor (IGF)–I and IGF binding proteins in female horses. *J Anim Sci* 1996; 74: 1936–1942.

[47]Roughead ZK, Lukaski HC. Inadequate copper intake reduces serum insulin–like growth factor–I and bone strength in growing rats fed graded amounts of copper and zinc. *J Nutr* 2003 ;133: 442–428.

[48]Watson ED, Bae SE, Thomassen R, Thomson SRM, Woad K, Armstrong DG. Insulin–like growth factors–I and –II and insulin–like growth factor–binding protein–2 in dominant equine follicles during spring transition and the ovulatory season. *Reprod* 2004; 128: 321–329.

[49]Sticker LS, Thompson DL Jr, Bunting LD, Fernandez JM, DePew CL, Nadal MR. Feed deprivation of mares: plasma metabolite and hormonal concentrations and responses to exercise. *J Anim Sci* 1995; 73: 3696–3704.

[50]Carlson D, Poulsen HD, Vestergaard M. Additional dietary zinc for weaning piglets is associated with elevated concentrations of serum IGF–I. *J Anim Physiol Anim Nutr (Berl)* 2004; 88: 332–339.

[51]de Moffarts B, Kirschvink N, Art T, Pincemail J, Lekeux P. Effect of oral antioxidant supplementation on blood antioxidant status in trained thoroughbred horses. *Vet J* 2005; 169: 65–74.

[52]Halliwell B, Gutteridge JMC. *Free radicals in biology and medicine*. 3rd ed. New York: Oxford university Press; 1999.

[53]Stabel JR, Spears JW, Brown TT Jr. Effect of copper deficiency on tissue, blood characteristics, and immune function of calves challenged with infectious bovine rhinotracheitis virus and Pasteurella hemolytica. *J Anim Sci* 1993; 71: 1247– 1255.

[54]Anastassopoulou J, Theophanides T. Magnesium/DNA interactions and the possible relation of magnesium to carcinogenesis. Irradiation and free radicals. *Crit Rev Oncol Hematol* 2002; 42: 79–91

[55]Prasad AS. Clinical, immunological, anti–inflammatory and antioxidant roles of zinc. *Exp Gerontol* 2008 ; 43: 370–377.

[56]Shenkin A. Serum prealbumin: Is it a marker of nutritional status or of risk of malnutrition. *Clin Chem* 2006; 52: 2177–9.

[57]Kirschvink N, de Moffarts B, Farnir F, Pincemail J, Lekeux P. Investigation of blood oxidant/antioxidant markers in healthy competition horses of different breeds. *Equine Vet J Suppl* 2006; 36: 239–244.

[58]Cymbaluk NF, Christensen DA. Copper, Zinc and Manganese Concentrations in Equine Liver, Kidney and Plasma. *Can Vet J* 1986; 27 : 206– 210.

[59]Yörük I, Deger Y, Mert H, Mert N. Serum concentration of copper, zinc, iron and cobalt and the copper/zinc ratio in horses with equine herpesvirus–1. *Biol Trace Elem Res* 2007; 118 :38–42.

[60]Dede S, Değer Y, Değer S, Tanrıtanır P. Plasma levels of zinc, copper,copper/zinc ratio, and activity of carbonic anhydrase in equine piroplasmosis. *Biol Trace Elem Res* 2008; 125: 41–45.

[61]Callewaert L, Michiels CW. Lysozymes in the animal kingdom. *J Biosci* 2010; 35: 127–160.

[62]Zhao J, Song L, Li C, Zou H, Ni D, Wang W, *et al*. Molecular cloning of an invertebrate goose–type lysozyme gene from Chlamys farreri, and lytic activity of the recombinant protein. *Mol Immunol* 2007; 44:1198–1208.

[63]Permyakov EA, Berliner LJ. α –Lactalbumin: structure and function. *FEBS Lett*2000; 473: 269–274.

[64]Bäck M, Hlawaty H, Labat C, Michel J–B, Brink C. The oral cavity and age: A site of chronic inflammation? *PLoS ONE* 2007; 12:e1351.

[65]Eggert–Kruse W, Botz I, Pohl S, Rohr G, Strowitzki T. Antimicrobial activity of human cervical mucus. *Hum Reprod* 2000; 15: 778–784.

[66]Song ZH, Hou S. A new analytical procedure for assay of lysozyme in human teat and saliva with immobilized reagents in flow injection chemiluinescence system. *Anal Sci* 2003; 19: 347–352.

[67]Katila T, Lock TF, Hoffmannl WE, Smith AR. Lysozyme, alkaline phosphatase and neutrophils in uterine secretions of mares with differing resistance to endometritis. *Theriogenology* 1990; 33 :723–732.

[68]Kmiliauskis MA, Palmeira P, Arslanian C, Pontes GN, Costa–Carvalho BT, Jacob CM, *et al*. Salivary lysozyme levels in patients with primary immunodeficiencies. *Allergol et Immunopathol* 2005; 33: 65–68.

[69]Yue WF, Li GL, Liu JM, Sun JT, Sun HX, Li XH, *et al*. Anti–oxidation and immune responses in mice upon exposure to manganese superoxide dismutase expressed in silkworm larvae, Bombyx mori L. *Cell Biol Internat* 2007 ; 31: 974–978.

[70]Lastra M, Pastein R, Camacho A, Monroy B, Aguilar AE. Zinc intervention on macrophages and lymphocytes response. *J Trace Elem Med. Biol* 2001; 15: 5–10.