Evaluation of Oral Multiminerals Supplementation for Treatment of Mineral Deficiency in Arabian Mares

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Abstract: The objective of the present study was to determine the effectiveness of mineral mixture in treatment of Arabian mares fed on mineral deficient diet. General performance, serum minerals; calcium (Ca), phosphorous (P) and magnesium (Mg), electrolytes; sodium (Na), potassium (K) and chloride (Cl), trace elements; selenium (Se), copper (Cu), iron (Fe), cobalt (Co) and zinc (Zn), protein profile, leptin hormone, blood leukocytes, immune status; phagocytosis of peripheral blood monocytes (PBM) and antibodies against Streptococcus equi and E. coli antigens, lysozyme activities as well as oxidative/antioxidant markers were evaluated. Five Arabian mares clinically suffering from suspected general manifestations of mineral deficiencies were treated with mineral mix orally at dose level of 15 ml/100kg body weight every other day for 12 weeks. Blood samples were collected before treatment (zero time) then every 4 weeks during the time of treatment. Results revealed that after mineral supplementation, concentrations of serum Ca, P, Na, Cl, Fe, Cu, Zn and Co increased significantly (P<0.01) to reach normal values. Total serum protein level, albumin (P<0.01) and globulin (P<0.05), were also increased reaching their normal values. Serum leptin concentration was decreased after mineral supplementation. Total leukocytic count, neutrophils and lymphocytes values were increased to restore normal values after mineral mix supplementation. Phagocytic percentage, phagocytic index of peripheral blood monocytes and serum lysozyme activity were markedly increased (P<0.05) after treatment. Levels of malondialdehyde (MDA) were decreased (P<0.05) during the period of treatment. Serum antioxidant capacity (TAC) was significantly increased after 8 weeks till the end of treatment. No significant change was recorded in serum nitrite. It could be concluded that a loose mineral mix in drinking water was an effective means of supplying macro and micro elements to Arabian mares. The animals appeared in a good shape with healthy body coat by the end of the 12th week of supplementation.

Key words: Minerals · Arabian mares horse · Leptin · Serum proteins · Leukogram · Immunity · Oxidative stress

INTRODUCTION

Minerals have several functions in the body, including the formation of the body structure, co-factor for enzymes and transformation of energy. They are constituents of the organic compounds such as proteins and lipids which make up muscles, organs, blood cells and other soft tissues of the body. Moreover, they are important in activation of enzyme and hormone systems [1]. Good health and performance in terms of growth, immunity, milk production and fertility, depend on adequate and balanced intake of macro and micro minerals. Ca, P, iodine (I), Cu, Mn, Se and Zn are needed for normal reproduction [2] and Ca, P, Mg, Co, I, Cu, Zn, Mn are needed for growth and proper bone development [1,3]. Harmonious functioning of the immune system requires the presence of several macro- and microelements. In animals, lack of Ca, Mg, Se, Fe, Zn, Cu and iodine have been associated with signs of immunodeficiency [4]. Availability of minerals in horses is closely related to mineral concentrations in their rations. Mineral contents of the feedstuffs may vary according to region, soil and

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plant types, vegetation phase and harvesting time of the plant [5,6] These variations may cause some problems in ration formulation to provide a stable intake of minerals.

Leptin is a polypeptide hormone, which is synthesized primarily by white adipose tissue and is secreted into the blood [7]. It is suggested that leptin is involved in the central and/or peripheral regulation of body homeostasis, energy intake, storage and expenditure, fertility and immune functions [8]. A marginal zinc deficiency could affect leptin secretion and serum leptin concentration which may contribute to hypogonadism in rats [9]. In the horse, leptin concentration was not only related to body condition but also to age, gender, feed intake and season [10-14]. During the winter season, mares in poor body condition and receiving restricted daily ration exhibited lower leptin levels and more prolonged anovulatory season compared to those in high body condition and affected by the same feed restriction [11,13].

Mineral deficiencies in mares may cause disorders in growth and development, besides decreased immune function or impaired reproductive efficiency [15]. Cu deficiencies have been shown to result in lowered bactericidal activities of blood leukocytes in cattle and sheep [16,17] and resulted in reduced neutrophil killing activity and decreased interferon production by mononuclear cells [18]. Zinc and copper-deficiency can negatively impact immune development and function [3]. Zinc deficiency has negative effects on growth rate, specific organ weights, hematological parameters and serum levels of Cu and Fe [19]. The ability of zinc to function as an antioxidant and membrane stabilizing agent suggests that it has a role in prevention of free radical inducing injury during inflammatory processes [20]. Zinc deficiency may cause delayed ovulation in mares [21] and compromises the function of T cells and several other immune cells [15]. Selenium stimulates the development and function of all types of white blood cells and enhances the ability of lymphocytes and killer cells. Selenium deficiency reduces the ability of bovine blood neutrophils to kill bacteria [22] and chemotactic migration of neutrophils [23].

Oxidative stress causes reactive oxygen species (ROS), as “free radicals” superoxide radical (O$_2^-$), hydroxyl radical (HO$^-$) and non-radicals; hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$) and hydrochlorous acid (HOCl) which are needed for proper function of the immune system [24]. Oxidants are essentially generated by metabolic enzymes, inflammatory cells and mitochondrial electron leakage and may -under certain conditions- have a pro-inflammatory stimulatory role. Excessive oxidant generation or antioxidant insufficiency can lead to oxidative stress. It can cause tissue damage and cell death by destroying cell proteins, DNA and fatty acids. This can lead to illness due to decrease in immune function. The role of antioxidants are scavenging free radicals, inhibiting excess ROS production and promoting repair of damaged tissues and cells. Trace elements, such as Se, Zn, Cu and Mn play an important catalytic role for the enzymatic activity of glutathione-peroxidase, GPx (Se) and superoxide dismutase; SOD (Zn, Cu, Mn). The catalytic activity of these enzymes allows the transformation of superoxide anion into hydrogen peroxide and water, thereby inactivating important amount of oxidants [24,25]. Nitric oxide production is an important microbiocidal mechanism of macrophages for inhibiting DNA synthesis, mitochondrial respiration and active transport in fungal and bacterial membrane [26].

The objective of the present study was to determine the effectiveness of mineral mixture as a means of supplying essential macro and micro minerals to Arabian mares fed on a deficient diet through investigation of the performance, serum minerals, protein profile, leukogram, leptin hormone, immunity as well as oxidative status before and after multi-mineral supplementation.

MATERIALS AND METHODS

Animals: This study was carried out on five Arabian breed non-lactating horse mares, 10 to 16 years of age and with a mean body weight of 270-330 Kg during the winter season (late December 2006 to April 2007). The animals were raised in a farm of horses at Cairo, Egypt. These animals were selected according of their general health condition. The mares were in a bad condition.

Clinical Examination: Examined mares were clinically suffering from general manifestations of mineral deficiency; emaciation, dry coat and alopecia in some cases. Body temperature, pulse and respiratory rate, posture and lymph nodes were normal. There were no specific symptoms among investigated animals and they were found free from external and internal parasites.

Feeding System: All mares were basically fed on barley as concentrates at level of 3.5 kg/head/day. In addition, animals were supplemented with Berseem clover (Trifolium alexandrinum) 10 kg/head/day and wheat
Table 1: Constituents Formula of mineral TS (oral solution + supplementary mineral diet)

<table>
<thead>
<tr>
<th>Contents</th>
<th>Amount per Liter</th>
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</thead>
<tbody>
<tr>
<td>Magnesium Hydroxide</td>
<td>30.00 g</td>
</tr>
<tr>
<td>Calcium Hydroxide</td>
<td>25.00 g</td>
</tr>
<tr>
<td>Zinc Oxide</td>
<td>3.26 g</td>
</tr>
<tr>
<td>Phosphoric acid diluted</td>
<td>270.00 ml</td>
</tr>
<tr>
<td>Citric acid</td>
<td>6.00 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>16.50 g</td>
</tr>
<tr>
<td>Copper Chloride</td>
<td>0.00 g</td>
</tr>
<tr>
<td>Manganese Chloride</td>
<td>8.79 g</td>
</tr>
<tr>
<td>Cobalt Chloride</td>
<td>0.100 g</td>
</tr>
<tr>
<td>Iron Chloride solution</td>
<td>15.00 ml</td>
</tr>
</tbody>
</table>

straw (tibn) as the roughage portion (0.5 kg/head/day). Common salt (NaCl) was added at level of 5g per kg ration. Fresh drinking water was offered ad libitum.

Dry and green rations offered to the animals were analyzed for dry matter (DM) and crude protein (CP) as well as macro and micro-elements contents according to the technique adopted by the Association of Official Analytical Chemists [27]. Neutral detergent fiber (NDF) was also determined [28].

Treatment with Mineral-Mix and Laboratory Examination: Animals were treated with minerals-mix and investigations were carried out to study its effects on some cellular and biochemical components of blood, immunity and reproductive performance of Arabian mares.

Animals received mineral-mix (Mineral TS-oral solution, supplementary mineral diet-Laboratories biove, 62510 Arques, France) orally at a dose level of 15 ml/100kg body weight mixed with water. Treatment was performed every other day for 12 weeks. Constituents of mineral-mix are illustrated in Table 1. Mares were clinically examined during the treatment period according to Radostitis et al. [29].

Blood Sampling: Samples were taken on day 0 (before initiation of treatment) and every 4 weeks for the successive 12 weeks. Three blood samples were collected by jugular puncture from each animal in the early morning (8:00 a.m) before ration was offered. The first blood sample was anticoagulated by disodium ethylene diamine tetra acetic acid (EDTA) and was used for evaluation of the leukogram. The second sample was received in a plain centrifuge tube, centrifugated at 3000 rpm for 10 min. and serum was then harvested and kept at -20°C until analysed for minerals, serum proteins, leptin hormone, humeral immunity, antioxidant and oxidative stress markers. Minerals and leptin were analysed before treatment and after 12 weeks only. The third blood sample was collected on heparin for estimation of cell mediated immunity.

Analytical Techniques
Biochemical and Hormonal Parameters
Macro and Micro-elements Assay: Serum calcium, inorganic phosphorus and magnesium were estimated using test kits supplied by bioMérieux-France according to Gindler and King [30], Goodwin [31] and Gindler [32], respectively. Sodium and potassium were estimated in serum by a Flame Photometer (Jenway, England Model PFP7). Serum chloride was estimated spectrophotometrically according to Skeggs and Hochstrasser [33] using kits supplied from Quimico Clinica Aplicada S. A.- Spain. Quantitative estimation of iron, copper and zinc was carried out using Atomic Absorption Spectrophotometer (Perkin Elmer Mod. 3300, USA) according to Fernandez and Kahan [34]. Serum cobalt and selenium were determined using Varian Spectra AA 220 Atomic Absorption Spectrophotometer equipped with a graphite furnace tube atomizer (GTA) for graphite furnace AAS, according to the methods of Blanchflower et al. [35] and Hoening [36], respectively.

Determination of Serum Proteins: Serum total proteins and albumin were analysed using test kits supplied by bioMérieux-France according to Henary et al. [37] and Doumas et al. [38], respectively.

Radioimmunoassay for Leptin: Serum leptin concentration was determined using multispecies leptin RIA kit (Linco Research, Inc, USA), according to Maffei et al. [39]. The sensitivity of the assay, intra- and inter-assay coefficients of variation were 1ng/ml, 2.8 and 8%, respectively.

Leukogram: Total white blood cell count and differential leukocyte count were determined according to Feldman et al. [40].

The Immune Response
Phagocytosis of Peripheral Blood Monocytes: Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation from fresh heparinized blood as described by Boyum [41] and McKeilve et al. [42]. In this technique, leucocyte rich plasma was prepared by erythrocyte sedimentation (20 minutes at room temperature, RT) and layered onto
Ficoll-paque 1.077 (Sigma). After centrifugation (654 g for 30 minutes at RT), PBMCs were removed, washed twice in sterile phosphate buffered saline (PBS, pH 7.4), centrifuged (450 g for 10 minutes at 4°C). The glass adherent macrophages were obtained according to Cheung et al. [43], by placing 1 ml of cell suspension (PBMCs; 10^6/ml) into cell culture and staining chambers (CCSC) containing sterile rounded cover slip and incubate for 1 hour at 37°C in 5% CO₂ and 90% humidity. The monolayer was washed to remove non adherent cells and reincubated over night in the same conditions. Then, 1 ml of Candida albicans (10^6/ml) was inoculated into the above prepared monolayer monocyte and incubated at the same conditions for one hour then washed 3 times, air dried, fixed and stained with Giemsa stain. Finally phagocyctic percentage and phagocyctic index were calculated.

**Humoral Immunity:** It was detected by measuring antibody titer against two famous bacterial diseases affecting equine using indirect ELISA. Streptococcus equi antigen was prepared according to Galan and Timoney [44], while E. coli lysate antigen (10 μg/ml) was purchased from Bio Richmond, CA, USA.

ELISA procedure was done according to Maghraby and Bahgat [45] with some modification. Indirect ELISA was used for detection of various antibodies against Streptococcus equi and E. coli. antigens. Each antigen was diluted in coating buffer, pH 9.6 while serum was diluted 1:50 in phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) and conjugated anti-equine IgG diluted 1:1,000. Orthophenyl diamine (OPD) diluted substrate solution was used. The plate was read using an automated ELISA reader set at 490 nm. Positive samples were those with optical density (OD) higher than the defined cut off 2 X mean OD of known negative samples.

**Lysozyme Activity Analysis:** Lysozyme activity was measured by the lysozyme assay method according to Peters and Vantranpen [46]. Heat-killed Micrococcus lypseuticus (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 standard dilutions of chicken egg white lysozyme from 0-500 μg/ml or with serum samples. Petri dishes were incubated at room temperature for at least 18 h. The diameter of the lysis zones around each well was measured and its area (mm) was calculated as the test result. The lytic zones were proportional to the concentration of lysozyme.

**Oxidant/antioxidant Markers Assessment:** Serum total antioxidant capacity (TAC) and lipid peroxidation product (Malonaldehyde; MDA) were measured using specific kits purchased from Bio-diagnostics, Dokki, Egypt.

Serum total antioxidant capacity (TAC) was determined according to the method of Koracevic et al. [47]. This determination is performed by the reaction of antioxidants in serum with a defined amount of exogenous hydrogen peroxide (H₂O₂). Antioxidants in the sample eliminate certain amount of the provided H₂O₂. The residual H₂O₂ is determined by an enzymatic reaction which involves the conversion of 3,5 dichloro-2-hydroxybenzensulphinate to a coloured product that is read at 505 nm.

Serum MDA was determined according to the method of Satoh [48]. This technique is based on thiobarbituric acid which reacts with MDA in acidic media at temperature of 95°C for 30 min. to form thiobarbituric reactive product. The absorbance of the resultant pink product can be measured at 534 nm.

Serum nitrite was estimated according to Rajaraman et al. [49]. Serum samples (100 μl) were mixed with an equal volume of freshly prepared Greiss reagent, incubated for 10 min. at room temperature and absorbency was measured at 570 nm using a micro titer plate reader. The nitrite level (μMol) in serum samples was calculated by comparing the optical density against the nitrite standard curve of sodium nitrite in distilled water.

**Statistical Analysis:** All data were subjected to statistical analysis including the calculation of the mean and standard error. Student t-test was used for the evaluation of data of serum minerals and leptin before and after treatment. Other data were tested for significance using one-way analysis of variance (ANOVA) followed by Duncan's test multiple range [50] using SPSS version 10 computer program.

**RESULTS**

Clinical examination of the investigated animals showed no signs of illness or toxicity during the period of administration of mineral mix.

Chemical analysis of the ration consumed by horses is presented in Table 2. Results showed that barley was deficient in Ca, P, Na, Fe, Cu, Zn and Mn, while Co and Se
were marginally deficient. Magnesium and K were within the normal levels in barley. Berseem clover was adequate in Ca, Mg, Na, K, Fe, Cu, Mn, Co and Se contents, while P and Zn were deficient.

### Biochemical and Hormonal Parameters

**Serum minerals' Profile:** Serum minerals' profile of mares before (zero time) and after 12 weeks of treatment is shown in Table 3. There was significant increase in values of Ca, P, Na and Co (p<0.05), Cu, Zn, iron (p<0.01) and chloride (p<0.001) after supplementation of mineral mix, compared with values before treatment. There were no significant differences in the values of Ca/P ratio, Mg, K and Se.

**Serum Proteins:** Serum total protein level exhibited gradual increase which was marked (P<0.01) at the 12th week. There was significant (P=0.01) increase in serum albumin at the 4th week of treatment and onwards till the end of the study. A gradual increase in values of serum globulin was noticed which was significant (P<0.05) at the 12th week of treatment. No significant difference was recorded in values of serum A/G ratio during treatment (Fig. 1).

**Serum Leptin:** Results revealed decrease in serum leptin concentration after mineral supplementation (Table 3).

**Leukogram:** Total white blood cell counts (WBC), neutrophils and lymphocytes were significantly (P<0.01) increased after mineral supplementation compared to values obtained before treatment (Fig. 2). Eosinophils,
Table 4: Phagocytic percentage and index and lysozyme activity in blood of Arabian mares before (zero time) and after supplementation of multiminerals for 12 weeks

<table>
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<th>8</th>
<th>12</th>
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<tr>
<td>Phagocytic (%)</td>
<td>64.0±4.97</td>
<td>77.0±4.37</td>
<td>80.6±4.73</td>
<td>84.3±3.05</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>1.29±0.04</td>
<td>2.79±0.09</td>
<td>2.91±0.07</td>
<td>2.64±0.07</td>
</tr>
<tr>
<td>Lysozyme activity (µg/ml)</td>
<td>127.6±11.65</td>
<td>205.3±5.48</td>
<td>180.0±10.75</td>
<td>173.6±17.93</td>
</tr>
</tbody>
</table>

Means with different superscripts in the same row are significantly different at P<0.05

Fig. 3: Absolute eosinophils and monocytes counts of Arabian mares before (zero time) and after multi-minerals supplementation for 12 weeks. Basophils and monocytes were not significantly increased after mineral supplementation (Fig. 3).

Fig. 4: Antibody levels (optical density; O.D) in serum of Arabian mares against antigens of E. coli and Streptococcus equi before (zero time) and after supplementation with multiminerals for 12 weeks as estimated by ELISA. Different letters above the same coloured columns are significantly different at P<0.05

The Immune Response
Phagocytosis of Peripheral Blood Monocytes: Phagocytic percentage and phagocytic index of peripheral blood monocytes revealed significant (P<0.05) increase after mineral supplementation from the 4th week till the end of treatment (Table 4).

Humoral Immunity: High antibody levels against E. coli were detected in serum of the examined animals which were decreased significantly after addition of mineral mix to mares feed. The antibody response against Strept. equi was moderate in the beginning then it declined later on (Fig. 4).

Analysis of Lysozyme Activity: A significant increase in lysozyme activity was noticed in all periods of treatment specially at the 4th week after mineral supplementation (Table 4).

Oxidants/antioxidants Markers: Serum total antioxidant activity began to increase significantly from the 8th week of minerals supplementation. Lipid peroxides (Malondialdehyde) were decreased significantly from the 4th week till the end of treatment. There was no significant difference in serum nitric oxide (Fig. 5).

DISCUSSION
The main diet provided to the investigated Arabian mares was Berseem clover and Barley. Chemical analysis showed that Berseem was good source of essential macro and micro-minerals except for P and Zn which were found
Fig. 5: Total antioxidant capacity; TAC (mM/l), lipid peroxides; MDA (nM/ml) and nitric oxide; NO (μM) levels in serum of Arabian mares before (zero time) and after supplementation with multiminerals for 12 weeks. Different letters above the same coloured columns are significantly different at P<0.05.

To be deficient. On the other hand, barely was poor in all minerals except for Mg and K which were at the NRC (National Research Council, 1989) recommended concentrations. Therefore, ration fed to the investigated animals were low in Zn and P levels than the standard requirements of 40 ppm Zn and 0.17-0.34% P in the diet of horses [51]. In addition, B. segetum clover was rich in Ca content which exceeds the NRC requirements of 0.24-0.61% in the diet of horses. Ca:P ratio was considerably in excess of 7:1. Excess of Ca has in turn a suppressing effect on the hormonal mechanisms stimulating Ca mobilization [52].

Excessive levels of Ca can increase the need for other minerals as P, Mg, Zn, Mn, Cu, I, Fe and possibly others. Therefore, it appears that mares need to be supplied by multi-minerals in order to meet the NRC requirements. Horses will usually drink 4.5-9 liter of water per 1 kg of feed intake. On this basis we chose to test a loose mineral mix received in drinking water as a means of supplying minerals to equine. It has been recorded that mild P deficiency occurred on pastures containing only 0.15% [53]. This was accompanied by retarded growth, unthriftness, infertility and low milk yield. The concentration of P in diet in the current study indicated a low level of P supply to mares and according to the NRC values less than 0.17% indicates a suboptimum P. This is supported by the lower concentration of inorganic P in blood serum. According to a previous study [52], values less than 3.5 mg/dl indicate a suboptimum P status. As a role, clinical signs occur when blood levels of P fall from the normal of 4.5 mg/dl to 1.5-3.5mg/dl. Levels may fall as low as 1mg/dl or less in sever clinical cases [52]. Marginal deficiency of P was sufficient to cause disturbance of pituitary ovarian axis without manifestation of deficiency symptoms resulting in ovarian inactivity [6,54]. Also infertility due to P deficiency can be produced when there is excess of Ca in ration and pasture that interfere with P metabolism [54].

The disturbed Ca to P ratio has a blocking action on the pituitary and consequently on ovarian action [55]. It has been reported that absorption of Ca and P was better from diets containing Ca: P ratio of 2:1 than that with 1:1 ratio [56]. Even a higher ratio has been suggested to be associated with infertility. An excessive intake of Ca either in the diet or water increase Mg requirement. Excess K may also increase Mg requirements which may be attributed to decrease absorption or increase excretion of Mg or both [52]. It appears that excess intake of Ca and K in diet of the present horses (from diet or therapy) may explain the non-significant elevation of Mg in serum of animals after treatment.

In the present study, zero time values (mg/dl) of Ca (8.53±0.35) and P (2.61±0.12) in serum of mares were lower than recorded reference values (Ca, 11.43±0.30 and P, 6.14±0.23) stated for Arabian horses [57]. After 12 weeks of minerals mix supplementation, values of Ca and P were significantly (p<0.05) increased to be within the normal values; 10.63±0.49 and 3.87±0.38 mg/dl, respectively.

There was significant increase in serum Ca, P, Na, Ca (p<0.05), Cu, Zn, iron (p<0.01) and chloride (p<0.001) after therapy with mineral mix compared with values before treatment. There were no significant differences in the values of Ca/P ratio, Mg, K and Se.

Initial serum Na (132.20±2.20 mEq/l) and Cl (89.43±0.93 mEq/l) values were lower than normal levels (Na, 137-150 and Cl, 90-104 mEq/l) stated for horses [58]. After 12 weeks of treatment, serum Na and Cl levels increased reaching to normal values. There is no change in serum K levels during treatment. Potassium along with Na and Cl occur in fluids and soft tissues of the body. All three minerals function in osmotic pressure and acid-base balance. The body has a continuous need for K, Na and Cl since it has very little storage capacity for them and excessive intake is rapidly excreted [3].

For trace elements, only Zn concentration in the provided ration was below published estimates of requirement. Recommended minimum concentrations
of trace elements in diet of horses are (mg/kg dry matter): Fe, 40-50; Cu, 10; Co, 0.10; Zn, 40; Mn, 40; Se, 0.1 [51].

Before treatment, serum values (µg/dl) of Fe was (193.60±3.38), Zn (78.7±3.28) and Co (5.30±0.40) which were lower than the levels recorded in normal Arabian horses; (Fe, 294.60±18.63, Zn, 88.35±1.96 and Co, 9.00-22.00 [57,59]. Serum Cu was in the normal level (55.64±3.45) stated for horses (48.00-92.00) [52]. After 12 weeks of treatment, serum Fe, Cu, Zn and Co levels significantly increased to be within the normal levels. It has been found that Cu concentration below 50 µg/dl as associated with poor performance [60]. A previous study, Lamand [61] recorded that optimal levels of Zn lay between 80-120 µg/100 ml and the threshold for deficiency was 70 µg/100ml plasma. A list of problems of Zn deficiency includes poor growth, defective healing of wounds, infertility and impaired immunity. Several enzymes need Zn as a molecular component. Zinc is needed for the development and maintenance of hair and skin. Zinc is an essential component of the cytosolic superoxide dismutase (SOD) enzyme which is a vital free radicals scavenger, the activity that protects cells from oxidative stress [62]. Zinc is also essential for utilization of vitamins A and E [63]. Zinc functions in the immune system through energy production, protein synthesis and stabilization of membranes against bacterial endotoxins, antioxidant enzyme production and antibody production [64]. A deficiency of Zn reduces thymus function and its important role in the immunological process [65].

Arabian mares suffering from mineral deficiency showed values of serum total proteins (7.04±0.11 g/dl), albumin (3.49±0.03 g/dl), globulin (3.55±0.11 g/dl) and A/G ratio (0.98±0.03) which were lower than the normal levels [66] (total protein, 9.50±0.16, albumin, 5.38±0.52, globulin, 4.12±0.30 and A/G ratio, 1.31±0.02). After 12 weeks of mineral supplementation, serum proteins levels were gradually increased but did not reach the normal reference values. Albumin and globulin increase may be due to Zn supplementation which regulates nucleic acid metabolism by its role on DNA and RNA polymerase thus controlling cell replication and synthesis of proteins [58].

Serum leptin concentration (2.40±0.33 ng/ml) recorded in deficient Arabian mares was high above the level (0.65±0.17 ng/ml) stated for mares in the same season [13]. After mineral supplementation, leptin levels were markedly decreased (1.26±0.24 ng/ml). It has been reported that mean concentrations of leptin in mares were greater in summer (2.48±0.17 ng/ml) compared to winter (0.65±0.17 ng/ml) [13]. Previous studies Gentry et al. [11], Ferreir-Dias et al. [67] recorded that deep seasonal aneustasis in mares was accompanied by low leptin levels. Reports showed that moderate zinc administration significantly increased glucose uptake and leptin secretion by adipose tissue in mice [68].

Leukocytes are known to play important roles in both inflammatory and immune responses [40]. In the present study, before supplementation with mineral-mix, values of total white blood cells (6.53±0.66 x10⁹/µl), neutrophils (3.13±0.34 x10⁹/µl) and lymphocytes (2.64±0.27 x10⁹/µl) were below the reference values stated for normal Arabian horses [57], (WBCs, 9.08±0.04, neutrophils, 3.90±0.02 and lymphocytes, 4.5±0.12 x10⁹/µl). After mineral supplementation, total WBCs, neutrophils and lymphocytes were markedly increased (p<0.01) to reach the normal levels. Eosinophils, basophils and monocytes were non significantly increased after mineral supplementation.

The examined Arabian mares showed high amounts of antibodies against E. coli and moderate amount of antibodies against Strept. equi. The results may be due to infection with these microorganisms and may indicate decrease in the mares immune response. It has been reported that deficiencies of specific nutrients can reduce immune responses and increase disease susceptibility [69]. For example, copper deficiency may result in decreased humoral and cell-mediated immunity, Zn deficiency impedes host defense systems leading to increased susceptibility to a variety of pathogens and iron deficiency results in impaired cell-mediated immunity [17,0]. Also, magnesium was considered as a natural cofactor for the conversion of complement factor C3 to C3b in the alternative pathway of complement activation [71].

In accordance with the previous results, phagocyte activity of monocytes as evaluated in vitro by phagocytic percentage and phagocytic index or in vivo as estimated by lysozyme amount significantly increased after mineral supplementation. The results indicate the immunological role of the provided minerals. Previous studies, Lastra et al. [72] recorded that there was a significant increment in the peritoneal macrophages phagocyte index and that the immunological role of zinc is most likely mediated by monocyte activation and cytokine release. Also, Wan-Fu et al. [73] added that copper, zinc, manganese and iron increase the activity of NK cell function.
A number of trace minerals are required for functioning of enzymes involved in the antioxidant defense system. Results of antioxidant activity (significant increase of TAC and decrease of MDA indicate decline in the oxidative stress by the antioxidant effect of the provided trace elements as Zn, Cu, Mg and Mn. The results are supported by many workers who reported that these elements reduced the free radicals and protect the cells by their antioxidant action [20,71].

In conclusion, a loose mineral-mix in drinking water was an effective means of supplying macro and micro elements to Arabian mares. By the end of the 12th week of supplementation, the animals appeared in a good shape with healthy body coat. Values of serum minerals serum minerals and proteins and blood leukocytes were restored toward the normal. Evaluation of the phagocytic activity of monocytes, serum antibodies, lysosomal activity, oxidant/antioxidant equilibrium indicate the positive immunological and antioxidant effects of mineral supplementation.

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