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Effects of Dietary Supplementation of Organic and Inorganic Zn, Cu and Mn on Oxidant/Antioxidant Balance in Laying Hens

Aziz BÜLBÜL* Tuba BÜLBÜL** Seher KÜÇÜKERSAN*** Meltem ŞİRELİ**** Abdullah ERYAVUZ*

* Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Physiology, Afyonkarahisar - TURKEY
** Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutrition Diseases, Afyonkarahisar - TURKEY
*** Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutrition Diseases, Ankara - TURKEY
**** Ankara University, Faculty of Veterinary Medicine, Department of Physiology, Ankara – TURKEY

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Summary

Effects of organic and inorganic zinc, copper and manganese supplementations into the diet on plasma malondialdehyde (MDA), nitric oxide (NO), glutation (GSH) and antioxidant activity (AOA) were investigated in totally 280 white strain laying hens (Lohmann LSL), aged 60 weeks. The experimental animals were divided into 7 different groups as 6 experimental and 1 control groups consisted of 40 laying hens in each group. Per group also divided into four subgroup containing 10 laying hens. The diets of the control and experimental groups contained mean 17% crude protein and 2750 kcal/kg metabolizable energy. The proteinate forms were used as organic sources of Zn, Cu and Mn, whereas the oxide forms were used as inorganic sources of Zn, Cu and Mn. 50 mg/kg of ZnO, 10 mg/kg of Zn-proteinate, 200 mg/kg of CuO, 50 mg/kg of Cu-proteinate, 30 mg/kg of MnO, 5 mg/kg of Mn-proteinate were added into the basal diets of ZnO, Zn-proteinate, CuO, Cu-proteinate, MnO and Mn-proteinate groups, respectively. Both organic and inorganic sources of Zn decreased plasma MDA and NO concentrations, while AOA remained unchanged, and whereas Zn-proteinate increased GSH concentration. CuO decreased the plasma MDA concentration but it did not alter the plasma NO, GSH and AOA. Cu-proteinate increased the plasma MDA concentration but decreased GSH and AOA. Both MnO and Mn-proteinate decreased the plasma MDA concentration and unchanged the plasma NO, GSH and AOA. It was concluded that both organic and inorganic sources of zinc and Mn decreased the oxidative stress in the laying hens, whereas especially organic copper increased this effect.

Keywords: *Zinc, Copper, Manganese, Lipid peroxidation, Antioxidant activity, Nitric oxide, Laying Hens*

Yumurtacı Tavuklarda Yeme Organik ve İnorganik Zn, Cu ve Mn İlavesinin Oksidan/Antioksidan Denge Üzerine Etkileri

Özet

Bu araştırma, yumurta tavuğu rasyonlarına organik ve inorganik çinko, bakır ve manganez katılmasının plazma malondialdehit (MDA), nitrik oksit (NO), glutasyon (GSH) ve antioksidan aktivite (AOA) üzerine etkisini belirlemek amacıyla yapılmıştır. Araştırmada 60 haftalık toplam 280 beyaz yumurta tavuğu (Lohmann LSL) kullanılmıştır. Araştırma her biri 40 tavuktan oluşan biri kontrol ve altı deneme grubu olmak üzere yedi grup halinde yürütülmüştür. Her grup 10 tavuk içeren 4 tekrar grubuna ayrılmıştır. Kontrol ve deneme grupları rasyonları ortalama %17 ham protein ve 2750 kcal/kg metabolize olabilir enerji içerecek şekilde düzenlenmiştir. Deneme gruplarından inorganik mineraller ilave edilenlere bu minerallerin oksit, organik ilave edilenlere ise proteinat formları eklenmiştir. Çinko oksit, Zn-proteinat, CuO, Cu-proteinat, MnO ve Mn-proteinat gruplarının yemlerine sırasıyla; ZnO 50 mg/kg, Zn-proteinat 10 mg/kg, CuO 200 mg/kg, Cu-proteinat 50 mg/kg, MnO 30 mg/kg ve Mn-proteinat 5 mg/kg ilave edildi. Araştırma sonunda organik ve inorganik Zn'nun plazma MDA ve NO düzeylerini azalttığı, AOA'yı değiştirmediği buna karşın Zn-proteinatın plazma GSH'ı artırdığı; CuO'in MDA düzeyini düşürürken NO, GSH ve AOA'yı değiştirmediği; Cu-proteinatın MDA düzeyini artırırken GSH ve AOA'yı azalttığı NO etkilemediği; MnO ve Mn-proteinatın plazma MDA düzeyini azaltırken NO, GSH ve AOA düzeylerini değiştirmediği belirlendi. Sonuç olarak organik ve inorganik Zn ve Mn'in yumurtacı tavuklarda oksidatif stresi baskılarken özellikle organik bakırın artırdığı belirlenmiştir.

Anahtar sözcükler: *Çinko, Bakır, Manganez, Lipid peroksidasyonu, Antioksidan aktivite, Nitrik oksit, Yumurta tavuğu*

İletişim (Correspondence)

Phone: +90 272 2281349/159
E-mail: abulbul@aku.edu.tr

INTRODUCTION

Minerals are generally added to diets of animals to ensure their nutritional requirements [1]. However, the bioavailability of minerals varies according to their sources [2]. The organic minerals have higher bioavailability than the inorganic minerals due to dissolution and absorption without reacting with the other minerals during the digestion [3]. Either the organic and inorganic forms of the trace minerals are commonly added into the diets of laying hens in order to improve their body strength, egg production and quality [1,4]. On the other hand, the inorganic mineral compounds can be easily oxidated throughout the digestive tract as they are free ions and extremely active and, form the complexes with the other substances in the diet preventing their absorption. Therefore, the chelates of the inorganic minerals with the organic compounds are commonly used in the animal nutrition in order to avoid the disadvantages of the inorganic minerals [3].

Oxygen radicals and other reactive oxygen species (ROS) are widely produced by cell metabolism [5]. ROS can react with double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides and, cause to tissue damage. However, there are some defense mechanisms to remove ROS in the cells. Trace minerals are involved directly or indirectly in the formation of many enzymes against ROS. For example, Zn is contributed to Zn/Cu superoxide dismutase (SOD) formation together with Cu [6]. In addition, Kraus et al. [7] found that Zn deficiency in rats decreased serum GSH concentration. The adding of Zn decreases the serum MDA levels in human [8], in rat [9] and in quail [10], while Zn deficiency increases it. Cu has redox effect which is essential for every living organism. Cu is involved in Cu/Zn-SOD and ceruloplasmin in human and rat and, the activities of these enzymes slow down in case of Cu deficiency [11]. Similarly, Mn is involved in the formation of Mn-SOD had an antioxidant activity [12].

The oxidant-antioxidant status can vary between mammalian and birds because the birds have higher levels of blood uric acid than mammalian [13]. Although the effects of minerals on lipid peroxidation and antioxidant defense system in mammalians and birds have been studied by many researchers [10,14]. Although there is available data on the effects of different minerals on oxidant and antioxidant status in laying hens, there is no data comparing the effects of oxide and proteinate sources of Zn, Cu and Mn on oxidant and antioxidant status in laying hens. Therefore, the objective of this research was to determine the MDA, NO, GSH concentrations and AOA levels in the laying hens fed with diet supplemented organic and inorganic forms of Zn, Cu and Mn.

MATERIAL and METHODS

Totally 280 white strain hybrid laying hens (Lohmann LSL), aging 60 weeks were used in the study and divided into 7 different groups, as six experiment and control group, each containing 40 laying hens. Furthermore, each group was divided into 4 sub-groups containing 10 laying hens. The formulation of basal diet is shown in *Table 1*. The basal diet was a typical laying hens diet containing 17% crude protein (CP) and 2750 kcal/kg metabolizable energy (ME) and was calculated to meet or slightly exceed the nutrients requirements recommended by the National Research Council. The basal diet contained 24.8 mg/kg Zn, 24.7 mg/kg Mn, and 9.8 mg/kg Cu.

Table 1. *Ingredient compositions of basal diet*
Tablo 1. *Temel yemin bileşimi*

Composition of basal diets*	%
Corn	45.30
Wheat	10
Soybean meal (%36 CP)	26
Meat-bone meal	3
Vegetable oil	3
Limestone	10
Dicalcium phosphate	2
Salt (NaCl)	0.25
Vitamin premix[1]	0.20
Mineral premix[2]	0.1
DL-Methionine	0.15
Calculated Analysis	
CP, %	17.10
ME, kcal/kg	2757
Calcium %	4.12
Phosphorus, %	0.83
Digestible phsophorus, %	0.59
Methionine + cystine, %	0.69
Lysine, %	0.95

[1] Rovimix 121 Provided per kilogram of diet: vitamin A 4.800.00 IU; vitamin D_3 1.000.000 IU; Vitamin E 8.000 mg; Vitamin K_3 1.600 mg; vitamin B_1 1.200 mg; vitamin B_2 2.400 mg; vitamin B_6 2.000 mg; vitamin B_{12} 8 mg; niacin 10.000 mg, Ca-D-Pantotenate 2.400 mg; folic acid 250 mg; choline chloride 100.000 mg.
[2] Remineral S Provided per kilogram of diet: Mn 70.000 mg; Mg 100.000 mg; Zn 60.000; Fe 25.000 mg; Cu 5.000 mg; Co 150 mg; Se 150 mg; Ca 100.000 mg; I 400 mg.
*The basal diet contained 24.8 mg/kg Zn, 24.7 mg/kg Mn, and 9.8 mg/kg Cu

The minerals were added into the basal diet of the experimental groups as declared in *Table 2*. The laying hens were fed on group basis, and the feed and water were offered "ad libitum" for daily consumption, and the duration of lighting was 17 hours a day including day light through the research. The study lasted 8 weeks.

Table 2. *The concentrations of organic and inorganic sources of Zn, Cu and Mn supplemented to the basal diet (mg/kg)*

Tablo 2. *Temel yeme katılan inorganik ve organik Zn, Cu ve Mn kaynaklarının yoğunluğu (mg/kg)*

GROUPS	ZnO	Zn Proteinat	CuO	Cu Proteinat	MnO	Mn Proteinat
Control	-	-	-	-	-	-
ZnO	50	-	-	-	-	-
Zn Proteinat	-	10	-	-	-	-
CuO	-	-	200	-	-	-
Cu Proteinat	-	-	-	50	-	-
MnO	-	-	-	-	30	-
Mn Proteinat	-	-	-	-	-	5

At the end of the research, the blood samples were collected from vena brachialis of 3 laying hens randomly chosen each sub-group. The plasma were obtained by centrifugation (3000 g, 10 min, +4°C) to determine the MDA, NO, GSH and AOA values. Plasma lipid peroxidation evaluated thiobarbituric acid reactive substances (TBARS), was determined according to Draper et al.[15], GSH by by the method of Beutler et al.[16] and AOA according to Koracevic et al.[17].

Determination of plasma nitric oxide concentrations: Plasma nitric oxide concentrations were determined according to the procedure of Miranda et al.[18]. Nitrate was reduced to nitrite with vanadium (III) and then nitrite level measured by using Griess reagents. Serial dilutions 0.5–200 µM of Na nitrate (Merck, Germany) were used as standards. The results were expressed as µmol/L.

Statistical analysis: The data obtained in this research was subjected to one way ANOVA as completely randomized design using the general liner models procedure of SAS software. Significant differences among the means were determined by using Duncan's multiple-range test at $p<0.05$.

RESULTS

The effects of the supplementation of organic and inorganic sources of Zn, Cu and Mn to the diet of the laying hens on the MDA, NO, GSH and AOA levels are shown in *Tables 3-5*. The supplementation of ZnO and Zn-proteinate to diet decreased both MDA and NO concentrations in the plasma, whereas only Zn-proteinate increased the GSH level. However, both of ZnO and Zn-proteinate did not affect the plasma AOA level in laying hens.

The supplementation of CuO to diet decreased the MDA concentration in the plasma of laying hens as compared with control and Cu proteinate supplementation groups. On the other hand, the supplementation of Cu proteinate increased significantly the MDA concentrations in the laying hens as compared with the control group. The plasma NO concentration was not affected by the supplementation of organic and inorganic copper sources, whereas the organic source of Cu decreased the plasma GSH concentration and AOA level in the laying hens. The supplementation of organic and inorganic forms of Mn decreased the MDA concentration as compared with the control group. There were no differences on the NO, GSH and AOA concentrations supplementation of organic and inorganic manganese to diet.

Table 3. *The effects of the supplementation of organic and inorganic Zn to the diet of the laying hens on plasma levels of the MDA (nmol/L), NO (µmol/L), GSH (µmol/L) and AOA (mmol/L) (n=12, ±SE)*

Tablo 3. *Yumurtacı tavukların yemine organik ve inorganic Zn ilavesinin plazma MDA (nmol/L), NO (µmol/L), GSH (µmol/L) ve AOA (mmol/L) düzeyleri üzerine etkisi*

Groups[1]	MDA	NO	GSH	AOA
Control	44.10 ±3.59^{a}	20.71 ±0.79^{a}	23.19 ±2.13^{b}	8.38 ±0.44
ZnO	28.53 ±1.32^{b}	17.99 ±0.70^{b}	24.61 ±1.14^{b}	7.29 ±0.62
Zn Proteinat	17.27 ±1.05^{c}	15.45 ±0.68^{c}	33.17 ±3.03^{a}	7.76 ±0.44

[1]Control: Basal diet-no additives,
ZnO: Basal diet + 50 mg/kg ZnO, Zn proteinat: Basal diet + 10 mg/kg Zn proteinat,
$^{a,b,c:}$ Differences among treatments in the same column are significant.

Table 4. *The effects of the supplementation of organic and inorganic Cu to the diet of the laying hens on plasma levels of the MDA (nmol/L), NO (µmol/L), GSH (µmol/L) and AOA (mmol/L) levels (n=12, ±SE)*

Tablo 4. *Yumurtacı tavukların diyetlerine organik ve inorganic Cu ilavesinin plazma MDA (nmol/L), NO (µmol/L), GSH (µmol/L) ve AOA (mmol/L) düzeyleri üzerine etkisi*

Groups[1]	MDH	NO	GSH	AOA
Control	44.10 ±3.59[b]	20.71 ±0.79	23.19 ±2.13[a]	8.38 ±0.44[a]
CuO	32.58 ±3.33[c]	20.06 ±0.90	25.33 ±1.66[a]	7.50 ±0.38[a]
Cu Proteinat	54.83 ±1.99[a]	21.19 ±1.02	16.26 ±1.71[b]	5.40 ±0.42[b]

[1]Control: Basal diet-no additives,
CuO: Basal diet + 200 mg/kg CuO, Cu proteinat: basal diet + 50 mg/kg Cu proteinat
[a,b,c:] Differences among treatments in the same column are significant.

Table 5. *The effects of the supplementation of organic and inorganic Mn to the diet of the laying hens on plasma levels of the MDA (nmol/L), NO (µmol/L), GSH (µmol/L) and AOA (mmol/L) levels (n=12, ±SE)*

Tablo 5. *Yumurtacı tavukların diyetlerine organik ve inorganic Mn ilavesinin plazma MDA (nmol/L), NO (µmol/L), GSH (µmol/L) ve AOA (mmol/L) düzeyleri üzerine etkileri*

Groups[1]	MDH	NO	GSH	AOA
Control	44.10 ±3.59[a]	20.71 ±0.79	23.19 ±2.13	8.38 ±0.44
MnO	7.24 ±0.10[b]	21.20 ±0.83	26.33 ±1.63	8.30 ±0.27
Mn Proteinat	7.54 ±0.16[b]	20.31 ±0.81	25.38 ±1.86	7.87 ±0.34

[1]Control: Basal diet-no additives,
MnO: Basal diet + 30 mg/kg MnO, Mn proteinat: Basal diet+ 5 mg/kg Mn proteinat.
[a,b:] Differences among treatments in the same column are significant.

DISCUSSION

The most important reactant in free radical biochemistry in aerobic cells is oxygen and its radical derivatives (superoxide and hydroxyl radical), hydrogen peroxide and transition metals. As lipids are prone to oxidation of unsaturated bonds, it is perhaps reasonable to advocate lipid peroxidation as a significant event in the development of membrane damage. One of the major secondary oxidation products of peroxidized polyunsaturated fatty acids is the MDA [5]. Since the MDA has been found elevated in various diseases thought to be related to free radical damage, determination of this biomarker has been widely applied as the most common approach for the assessment of lipoperoxidation in biological and medical sciences[17,19]. In this study, the supplementation of both organic and inorganic zinc sources to the diet of laying hens decreased the MDA concentration. This result might be attributed to Cu/Zn-SOD because previous studies have showed that zinc and copper together involved in formation of Cu/Zn-SOD and the level of this enzyme elevated in case of supplementation of those minerals into diet [6,9]. In addition, Cu/Zn-SOD decreases lipid peroxidation by transforming superoxide (O^{-}) into H_2O_2 and dioxygen [12]. However, MDA concentration was more decreased by the organic Zn source than inorganic one. This was in accordance with the finding of Szentmihalyi et al.[2] who observed that organic zinc that traps free radicals better than the inorganic forms as *in vitro.*

Copper contributes to the formation of the two antioxidant enzymes; Ceruloplasmin and Cu/Zn-SOD [11]. A number of studies in the birds demonstrated that inorganic copper addition into the diets at the level of 13.2 mg/kg increased the Cu/Zn-SOD levels in liver, red blood cells [20], kidney, heart and blood plasma [21]. However, Zhang et al.[22] found that, Cu overload in rats increased the serum MDA concentration due to a remarkable drop in Cu/Zn-SOD and Se-GSH peroxidase activities in blood and a significant increase in hepatic Cu. In the present study, the inorganic source of Cu decreased the MDA concentration but the organic source of it increased the MDA concentration. This is probably due to the diversity of absorption of Cu in different Cu sources [3] because the diets used in this study contained similar amounts of Cu. Our results indicated that organic Cu amount used in this study increased lipid peroxidation, demonstrated to oxidative damage.

In the present study, both of organic and inorganic sources of Mn decreased MDA concentration in the blood. This might be due to

contribution of Mn to both the formation of Mn-SOD, which is a mitochondrial enzyme and traps some important oxidants, and the formation of Mn-prophirine [12]. Mn-prophirine is also known to have antioxidant activity by capturing superoxide, hydrogen peroxide, nitric peroxide and lipid peroxydes [23]. In addition, Trostchansky et al.[24] reported that uric acid increases the antioxidant activity of Mn-prophirine in the blood.

Nitric oxide has many physiologic functions and physio-pathologic effects in the organism and, synthesized by the enzymes iNOS/nNOS/eNOS from L-arginine. The half-life of NO is very short and, peroxynitrite ($OONO^-$) is formed by radical O^- following the reduction of NO_2 to NO_3. Nitric oxide is accepted as an antioxidant by capturing radical O^- and as an oxidant by forming peroxynitrite [25]. Our results showed that the supplementation of both organic and inorganic Zn to the diet decreased the NO level, whereas the adding of Cu and Mn to the diet did not affect it. There are different reports on the effect of minerals on NOS enzymes. David and Janore [26] found that zinc suppressed the enzyme iNOS. Bianchini et al.[27] reported that, although Cu-ceruloplasmin is an eNOS inhibitor, the iNOS synthesizes more NO from L-arginine than the eNOS. On the other hand, Cu/Zn-SOD and Mn-SOD remove radical super oxide and, this leads to an increase in the half-life of NO [28]. In addition, Pfeiffer et al.[29] suggested that Mn-porphirine is a direct inhibitor of cyclic guanylate cyclase and NO. In the present study, while this effect of Zn on NO is in agreement with previous results of David and Janore [25], the effect of Cu and Mn on NO in laying hens might be attributed to difference of minerals on efficiency of NOS.

Glutathione is known to react directly with ROS and acts as a non-enzymatic antioxidant [16]. AOA is not only a sum of antioxidants but also a dynamic antioxidant defense system developed against currently present oxidants [17]. The supplementation of Zn to the diet removes the radical superoxide by increasing the levels of SOD and catalase and, this mechanism prevents the autooxidation of GSH and thiols. In addition, the reduced GSH gen release increases in cell exposured to the hydrogen peroxide [30]. In this study, the increased GSH in Zn-proteinate group, but not ZnO group, can be explained by removing of radical O^- by increment of other antioxidant enzymes such as SOD and catalase, thus this mechanism indicates that zinc protects GSH in the blood. However, the unchanged levels of GSH in ZnO group might be due to low bioavailability of zinc in the ZnO_3. In addition, the supplementation of both organic and inorganic zinc sources to diet unchanged AOA, indicated defense system against lipid peroxidation.

Gaetke and Chow [27] reported that oxidative damage has been linked to Cu- overload or exposure to excess Cu. Inorganic Cu source used in this study unchanged the GSH, whereas organic Cu source decreased it. This result can be attributed to higher bioavailability of Cu in the Cu-proteinate. In addition, the decreased AOA in laying hens fed with supplemented organic copper source supported this suggestion. Our results showed that addition of manganese to the diet of hens had no effect on plasma GSH and AOA levels. This can be attributed to extremely low levels of lipid peroxidation in this study.

As a result, the lipid peroxidation is decreased by the supplementation of the inorganic and organic sources of zinc and manganese, and inorganic copper to diet. Therefore, we concluded that both organic and inorganic sources of zinc and Mn decreased the oxidative stress in the laying hens, whereas especially organic copper source increased it.

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