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Influence of organic selenium supplementation on the accumulation of toxic and essential trace elements involved in the antioxidant system of chicken

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6 **and essential trace elements involved in the antioxidant system of chicken**
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Abstract

The aim of the study was to investigate the interactions between selenium (Se) and some trace elements, both toxic and essential, which are all involved in the antioxidant system. A total of 128 day-old chicks (*Gallus gallus*, broilers) were used to investigate the effect of Se yeast supplementation on the accumulation of cadmium (Cd), copper (Cu) iron (Fe) and zinc (Zn). There were 4 replicates of 4 dietary treatments: T1 (basal diet with no added Se, analyzed to contain 0.21 mg kg⁻¹), T2 (T1 with 0.15 mg kg⁻¹ Se added), T3 (T1 with 0.3 mg kg⁻¹ Se) and T4 (T1 with 3.0 mg kg⁻¹ Se). At 4th and 6th week, 2 chickens per replicate pen were sacrificed for whole blood, breast muscle and liver sample collections. Samples were analyzed by ICP-MS. Supplementation with Se-yeast, not only increased Se concentration but also reduced Cd concentration in the tissues. Selenium was negatively correlated with Cd and positively correlated with Zn, Cu and Fe. Cadmium was negatively correlated with Zn and Cu. Zinc was positively correlated with Cu. Iron was negatively correlated with Cu and uncorrelated with Zn and Cd. The balance between Se, Cu, Fe and Zn is important for proper antioxidant defence since they are integral part of various antioxidant enzymes.

Introduction

Trace elements are minerals that are needed in minute quantities for the proper growth, development, and physiology of the organism. In living animal organisms, trace elements are bound to various ligands in cellular membranes, cytoplasm and blood. They are transported and stored via several proteins including but not limited to albumin, ceruloplasmin, transferrin and metallothioneins which are often non-specific and so may be sites of interaction between trace elements (Serra-Majem et al. 2009; Southgate et al. 1989; Strachan 2010). They can be classified as essential including iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) and selenium (Se), probably essential nickel (Ni), vanadium (V) and cobalt (Co) both required in the diet, but that may have toxic effects at supraoptimal concentrations (Underwood and Suttle 1999) and potentially toxic including but not limited to aluminum (Al) arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg) not required in the diet, causing chronic negative effects at low concentrations and lethal effects at high concentrations (Bires et al. 1995; Uluozlu et al. 2009). The absorption, accumulation and toxicity of each metal may be affected by diverse factors, including interactions with other metals. Indeed, interactions between toxic and essential metals are central to mineral balance and the antioxidant defence system (López-Alonso et al. 2007; Pappas et al. 2010a; Pappas et al. 2010b).

Selenium is a trace element that plays a key role in the antioxidant defence system. It is the integral part of at least 25 selenoproteins and via their actions protects the organism from harmful actions of free radicals (Pappas et al. 2008). Copper and Zn are two of the most abundant trace elements found in the human body and are involved in the metabolism of oxygen and the biochemistry of redox reactions (Klotz et al. 2003). The enzyme CuZn-superoxide dismutase (SOD) catalyzes the dismutation of superoxide, which is constantly formed during aerobic metabolism, to oxygen and hydrogen peroxide. Copper and Zn are joined in cellular defense against oxidants by Se to form a triad of trace elements that are involved in cytosolic antioxidant defense: hydroperoxides, including H_2O_2 , are reduced to the respective alcohols or water in a reaction that is catalyzed by the selenoenzyme glutathione peroxidase (GPx) with glutathione (GSH) as the electron donor (Klotz et al. 2003). Similarly to Cu and Zn, iron's ability to accept or donate electrons makes it central to the catalytic activity of many redox-active enzymes. Iron is an integral part of several classes of enzymes, including cytochromes, enzymes involved in the synthesis of steroid hormones, detoxification of foreign substances in the liver, synthesis of neurotransmitters and DNA synthesis and

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3 breakdown (Gambling et al. 2008). Cadmium disrupts the antioxidant enzyme system,
4 resulting in increased oxygen radical production, which causes damage to membranous
5 structures such as mitochondria and endoplasmic reticulum (Schrauzer 2009). Cadmium is
6 known to induce the production of nitric oxide (NO) and reactive oxygen species (ROS) such
7 as hydroxyl radicals (OH^\bullet), superoxide anions radicals ($\text{O}_2^{\bullet-}$) and H_2O_2 . Furthermore, Cd
8 interferes with antioxidant enzymes, causes enzyme dysfunction and lipid peroxidation
9 (Ognjanovic et al. 2010).
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16 Essential trace metals may modify health risks from exposure to nonessential toxic
17 metals. A well known example of this is the reduction of Cd toxicity after pre-treatment with
18 Zn or the simultaneous administration of Zn with Cd (Jemai et al. 2007; Webb 1979). On the
19 other hand, the ingestion of a diet that is deficient in a particular essential element can
20 enhance the accumulation and toxicity of some toxic metals. Calcium, Fe and Zn deficiency,
21 for instance, enhance susceptibility to Cd and Pb toxicity (Goyer 1997; Jihen et al. 2008;
22 WHO 1996). Additionally, it has been demonstrated that toxic metals can disrupt trace
23 element metabolism. Cadmium toxicity affects Ca metabolism either by direct toxicity to
24 bone or indirectly from renal toxicity (Goyer 1997; Pappas et al. 2010a; Webb 1979).
25 Interactions between metals can occur at levels of exposure well below those at which toxicity
26 may be detected. In addition, domestic animals have the advantage that they are an important
27 source of food for humans and good biomonitor species for metal contamination worldwide
28 (Lopez Alonzo et al. 2004; Petersson-Grawe et al. 1997; Tuzen and Soylak 2007).
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39 The present study was part of a project designed to assess the effect of the inclusion of
40 Se in broiler's diet on the gene expression of antioxidant enzymes, trace element interactions
41 and Se impact on fatty acid composition and oxidative stability of lipids in meat. Previously,
42 we evaluated how Se affected gene expression of Se dependent and non-Se dependent
43 antioxidant enzymes (Zoidis et al. 2010). The aim of the present study was to investigate the
44 interactions between Se and some trace elements, both toxic and essential, which are all
45 involved in the antioxidant system. Therefore, the concentration of Se, Cd, Cu, Fe and Zn and
46 their interactions were assessed in blood, liver and breast muscle tissues of 4- and 6 week old
47 broilers fed diets supplemented with Se-yeast.
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Materials and methods

Animals, Diets and Design

Details of the genotype, nutrition and husbandry of the broilers used in this study have been described previously (Zoidis et al. 2010). In brief, a total of 128 day-old Cobb chickens (broilers) were used. The chickens were obtained from a commercial hatchery. All chickens had the same Se-status because the broiler breeder flock was fed a commercial diet with 0.20 mg kg⁻¹ Se. There were 4 replicate pens of 4 dietary treatments namely T1, T2, T3 and T4 randomly allocated in the house. Pen was the experimental unit. There were 8 chickens per pen, 32 per treatment. The study was designed considering that a concentration of 0.15 mg Se per kg of diet is recommended by the United States National Research Council (NRC 1994), that the maximum allowed Se inclusion level in the United States is 0.3 mg kg⁻¹ (Payne et al. 2005) and that current total maximum EU authorized level of Se in complete feed is 0.5 mg kg⁻¹. In T1, chickens were fed a basal diet without Se supplementation; the only Se present was that of feed ingredients (Table 1). In treatments T2, T3 and T4, the chickens were fed the same basal diet supplemented with additional Se from a yeast source, Sel-Plex[®] (Alltech Inc, Nicholasville, KY, USA) to provide 0.15, 0.3 and 3 mg kg⁻¹ Se respectively. Recent findings regarding chemical or enzymatic yeast protein hydrolysis revealed that selenomethionine accounts for 90% of total Se, while selenocystine, γ -glutamylselenocysteine, selenocystathione, methylselenocysteine and other compounds represent the rest of the total Se (Schrauzer 2006).

The duration of the study was 6 weeks with housing and care of chickens to conform the guidelines of the Faculty of Animal Science and Aquaculture of the Agricultural University of Athens. The chickens were raised, according to Cobb's management manual, in a house where light and ventilation were controlled. The chickens were fed a starter diet to the 2nd wk of their life, a grower diet to the 4th wk and a finisher diet thereafter. Feed and water were provided *ad libitum*. Each growing phase diet (starter, grower and finisher) contained the same level of added Se which was the appropriate for each experimental treatment (Table 1). The lighting program was 23 hours of light and one hour of dark. Stocking density was according to EU legislation. At weekly intervals, chickens were weighted and the body weight was recorded. Furthermore, feed intake was measured weekly and feed to gain ratio was calculated. At the end of the 4th and 6th week, two chickens per pen were sacrificed by neck dislocation and liver, breast muscle and whole blood samples were collected for determination of trace elements concentration.

Trace element determination

The trace element concentrations were determined using inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, Elan 9000, PerkinElmer Life and Analytical Sciences Inc, Waltham, MA, USA). The instrumental parameters of the equipment used were: Nebuliser flow 0.775 L/min, Vacuum pressure 1.5×10^{-5} torr, Lens Voltage 950 W, Analogue Stage Voltage -1900 V, Pulse Stage Voltage 950 V, Sweeps/reading 20, Readings/replicate 1, Number of replicates 3, Time per sample 83 s. Feed samples were collected before feeding and milled before analysis through a 1 mm sieve (Cyclotec, 1093 sample mill, Tecator, Höganäs, Sweden). Initially, a complete digestion of the samples was performed with a microwave digestion system (CEM, Mars X-Press, NC, USA). Samples (0.5 g) of wet tissue or feed were soaked in 10 ml concentrated HNO_3 (65% w/v, Suprapur, Merck, Darmstadt, Germany). The samples were heated in the microwave accelerated digestion system according to the following program: the power was ramped during 20 min from 100W to 1200W and held for 15 min. The temperature reached a maximum of 200°C and followed by a cool down cycle for 15 min. Losses of volatile element compounds do not occur as the tubes are sealed during heating. The samples were then filtered with disposable syringe filters (Chromafil, Macherey-Nagel, Duren, Germany) and diluted 50 times with reversed osmosis water (Milli-Q Water Purification Systems, Billerica, MA, USA) before injection in the ICP-MS instrument. Standard solutions used for calibration curves were prepared from high purity standards (Multielenment standard solution, Fluka Analytical, Sigma-Aldrich, St Louis, USA). The analytical procedure was validated using a Se recovery procedure (Georgiou and Koupparis 1990) and a standard reference material (NIST-RM 8414 -bovine muscle powder-LGC Standards Promochem, Wesel, Germany). The Se recovery procedure was as follows: 4 samples from the same liver tissue (1 g each) were spiked with 250, 500 and 750 μl of Se standard solution of 4 mg kg^{-1} , respectively. The spiked samples were analyzed after the aforementioned analytical procedure. The recoveries of the procedure used to validate ICP-MS were in the range of 92.1-105.6 % indicating the accuracy of the method. Regarding the NIST-RM, certified and determined trace element concentrations are presented in Table 2.

Statistical analysis

The statistical analysis was performed using SPSS for Windows (Chicago, IL, USA). The data were analyzed using a multivariate linear model where Se, Cd, Zn, Cu and Fe concentration represented the response variables while treatment, age and tissue were the fixed factors. We tested the hypothesis that the trace element variations are correlated using

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3 Bartlett's test of sphericity and by calculating the pairwise Pearson correlations. Both
4 methods indicated that the concentrations are indeed correlated, which also reflects biological
5 plausibility. Hence, a multivariate analysis appeared to be necessary. The response variable
6 (Y) was approximately normally distributed as verified by employing both histograms and
7 normality tests. Thus using a multivariate linear model seemed like a reasonable approach.
8
9 The model was:

$$Y = \text{intercept} + \text{age} + \text{tissue} + \text{treatment} + (\text{age} \times \text{tissue}) + (\text{age} \times \text{treatment}) + (\text{tissue} \times \text{treatment}) + (\text{age} \times \text{tissue} \times \text{treatment}),$$

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19 where Y denotes Se, Cd, Zn, Cu and Fe concentrations.

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21 A particular aspect of our analysis was the comparison of the element correlations
22 before and after running the model. In this way, potential differences (or lack thereof) may be
23 revealed, which could otherwise be confounded due to ignoring the factor influence. The
24 statements of significance presented in this study were based on $P \leq 0.05$ unless otherwise
25 stated. In the Tables, the data are presented as the mean \pm SE of each of the main effects in
26 turn, pooled for all other main effects. Furthermore, in the results section all statistically
27 significant interactions are presented.
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Results

Supplementation of diet with 150, 300 and 3000 $\mu\text{g Se kg}^{-1}$ significantly ($P<0.001$) increased the Se concentration (data pooled for age and tissue effects) by a factor of 1.75, 2.1 and 8.6 respectively compared to the Se concentration of broilers fed the Se unsupplemented diet (Table 3). Overall, the concentration of Se in 6-week old broilers was lower compared with its concentration in 4-wk old broilers (data pooled for treatment and tissue effects, Table 3). The significant age X treatment interaction ($P<0.001$) revealed that Se concentration decreased with age for all four treatments. Tissue type significantly affected Se concentration ($P<0.001$, data pooled for age and treatment to exacerbate tissue effects, Table 3). Tissue Se concentration in descending order was liver>breast muscle>blood and this pattern was noted for all treatments as indicated by the significant treatment X tissue interactions ($P<0.001$). The age X tissue interaction ($P<0.001$) indicated that Se concentration in blood (pooled for all treatments) decreased as the age of broilers increased (489 ± 12.7 vs. $379\pm 12.7 \mu\text{g kg}^{-1}$). The same was evident in breast muscle (825 ± 14.2 vs. $756\pm 12.7 \mu\text{g kg}^{-1}$). However, Se concentration in liver increased as the age of broilers increased (1313 ± 12.7 vs. $1359\pm 12.7 \mu\text{g kg}^{-1}$). The significant treatment X age X tissue interactions ($P<0.001$) indicated that, although overall increasing age reduced Se concentration, some treatments for some tissues responded different with increasing age. In detail, for broilers fed diet T2, liver Se concentration did not differ as age progressed (784 ± 25.5 vs. $787\pm 25.5 \mu\text{g kg}^{-1}$). The same was true for liver Se concentration of broilers fed the T3 diet (869 ± 25.5 vs. $887\pm 25.5 \mu\text{g kg}^{-1}$). However, liver Se concentration of broilers fed T4 increased with age (3090 ± 25.5 vs. $3271\pm 25.5 \mu\text{g kg}^{-1}$). Finally, liver Se concentration of broilers fed T1 reduced with age (115 ± 25.5 vs. $88\pm 25.5 \mu\text{g kg}^{-1}$).

Selenium supplementation significantly ($P<0.001$) affected the concentration of Cd in the examined tissues (Table 3). In detail, Cd concentration in broiler tissues (pooled data) of treatments T2, T3, T4 was reduced by 41%, 37% and 13% respectively compared to the concentration of treatment T1. Cadmium concentration in blood was 19.8 times greater ($P<0.001$) than that in breast muscle while that in liver was 4.9 times greater than that in breast muscle. The present study revealed significant interactions between treatment and tissue regarding Cd accumulation. In detail, Cd concentration in blood of broilers fed diets T2 ($21.3\pm 0.86 \mu\text{g kg}^{-1}$) and T3 ($23.8\pm 0.86 \mu\text{g kg}^{-1}$) was lower than that of broilers fed Se unsupplemented diets ($37\pm 0.86 \mu\text{g kg}^{-1}$). Cadmium concentration was greater in 6-wk old broilers compared with its concentration in 4-wk old broilers. Furthermore, the age of broilers affected the Cd concentration in the tissues as revealed by the age X tissue interactions

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3 (P=0.001). In detail, 6-wk old broilers had higher Cd concentration in blood compared to 4-
4 wk old broilers (38 ± 0.6 vs. 21 ± 0.6 $\mu\text{g kg}^{-1}$). The treatment X age interaction (P<0.001) noted
5 in the present study indicated that the increment of Cd concentration with age differed
6 between treatments. In detail, Cd concentration in tissues of 4-wk vs. 6-wk old broilers fed
7 diets T1, T2, T3 and T4 was 9.7 ± 0.71 vs. 23.5 ± 0.71 , 9.4 ± 0.71 vs. 10.2 ± 0.71 , 9.2 ± 0.71 vs.
8 11.6 ± 0.71 and 8.9 ± 0.82 vs. 20.1 ± 0.71 $\mu\text{g kg}^{-1}$ respectively. Furthermore, there was a
9 significant (P=0.011) treatment X age X tissue interaction regarding Cd accumulation. In
10 detail, Cd concentration in the liver of broilers fed diets without added Se increased as the age
11 progressed (5.7 ± 1.23 vs. 16.4 ± 1.23 $\mu\text{g kg}^{-1}$ for 4-wk vs. 6-wk old broilers respectively).

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Iron concentration tended (P=0.078) to be increased in the tissues of broilers fed Se
supplemented diets (Table 3). Tissue type significantly affected Fe concentration (P<0.001).
In particular, blood was the tissue with the highest concentration of Fe. Iron concentration in
blood was 391203 ± 8442 $\mu\text{g kg}^{-1}$ followed by the concentration in liver (199317 ± 8442 $\mu\text{g kg}^{-1}$)
and breast muscle (5985 ± 8455 $\mu\text{g kg}^{-1}$). Overall, Fe concentration in broilers' tissues did
not differ with age, however, the noted age X tissue interaction (P<0.001) revealed that blood
Fe concentration decreased as the age increased (421838 ± 11940 vs. 360568 ± 11940 $\mu\text{g kg}^{-1}$).
Furthermore, the treatment X tissue interactions (P<0.05) showed that broilers fed the T2
(425865 ± 16885 $\mu\text{g kg}^{-1}$) and T3 (425702 ± 16885 $\mu\text{g kg}^{-1}$) diet had greater concentration of Fe
in the blood than broilers fed the T1 (360502 ± 16885 $\mu\text{g kg}^{-1}$) or T4 (352742 ± 16885 $\mu\text{g kg}^{-1}$)
diets.

Zinc concentration in tissues was not affected by Se addition to broiler diets (Table 3).
Zinc concentration was 6.6-fold greater in the liver and 1.4-fold greater in the breast muscle
than its concentration in the blood. In detail, Zn concentration in 6-wk old broilers was lower
compared with its concentration in 4-wk old broilers (P=0.034). Furthermore, the age X tissue
interactions (P=0.01) indicated that 6-wk old broilers had lower Zn concentration in blood
compared to 4-wk old broilers (4204 ± 346 vs. 3571 ± 346 $\mu\text{g kg}^{-1}$). The treatment X age X
tissue interactions (P<0.05) indicated that as the age of broilers progressed, the Zn
concentration in the blood and liver of broilers fed Se unsupplemented diets decreased
(4628 ± 691 vs. 3099 ± 691 $\mu\text{g kg}^{-1}$ for blood; 4917 ± 691 vs. 4510 ± 691 $\mu\text{g kg}^{-1}$ for liver).

Copper concentration was not affected by Se addition (Table 3). Liver contained
higher (P<0.001) concentration of Cu compared to that of blood and breast muscle. There was
a tendency (P=0.064) for 6-wk old broilers to contain more Cu than the 4-wk old ones.
Although Se supplementation did not affect Cu accumulation, the treatment X age X tissue

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3 interaction ($P=0.005$) showed that Cu concentration in the liver of broilers fed Se
4 unsupplemented diets decreased along with age (4092 ± 123 vs. $3170\pm 123 \mu\text{g kg}^{-1}$).
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7 The correlations between the trace elements before and after the application of the
8 model are presented in Table 4. After the application of the statistical model, the discernible
9 points by inspecting the results were that Se and Cu were correlated with all examined trace
10 elements; Zn was correlated with all examined trace elements except for Fe, and Cd correlated
11 with all examined trace elements except for Fe. In addition, the value and significance of
12 some pairwise correlations changed by incorporating the factors' influence (Table 4).
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15 Specifically, Se was negatively correlated with Cd and this relationship became
16 stronger after model application. Similarly, Se correlation with Zn was and remained strongly
17 positive. Likewise, Se correlated positively with Cu and remained so post-model. In contrast,
18 Se and Fe appeared uncorrelated in the descriptive statistics, while in fact they were found to
19 correlate positively when the examined factors were taken into account (Table 4). Hence, this
20 association was masked by ignoring the considered factors.
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23 Cadmium was negatively correlated with Zn and this relationship remained so, albeit
24 weaker after the model application. Furthermore, Cd and Cu were negatively correlated and
25 continued to be so post-model. The application of the model revealed that the seemingly
26 strong correlation between Cd and Fe was spurious as the two elements were in fact
27 uncorrelated (Table 4).
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30 Zinc and Cu were strongly positively correlated and their association remained
31 positive although not as profound. Zinc and Fe remained uncorrelated throughout the analysis.
32 Finally, Cu and Fe seemed initially uncorrelated, but taking the factors into account exposed a
33 weak negative correlation (Table 4).
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37 Discussion

38 This study revealed that supplementation of broilers' diet with Se, not only increased
39 Se concentration but also reduced Cd concentration in the tissues. Cadmium is absorbed from
40 the gastrointestinal tract and the lung and is mainly accumulated in liver and kidney where it
41 is bound to metallothionein (MT), a low molecular weight cysteine-rich, intracellular protein
42 with high affinity for both essential and non-essential metals (Klaassen et al. 1999). When Cd
43 concentration exceeds the binding capacity of MT, the non-bound Cd causes toxicity possibly
44 due to free radical induction and lipid peroxidation (Sarkar et al. 1997). The administration of
45 Se lowers the toxicity of Cd by shifting the distribution of tissue Cd from MT towards high-
46 molecular-mass proteins (Underwood and Suttle 1999; Wangher 2001). The reduction of Cd
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3 concentration noted in the present study may be attributed to the formation of Se-Cd
4 complexes. Indeed, addition of Se compounds has been proven an efficient therapy against
5 metal toxicity in mammals (Glynn et al. 1993), vegetables (Shanker et al. 1996) and fishes
6 (Paulsson and Lundberg 1989). Although only Se-yeast was examined in this study, inorganic
7 Se has been shown to be effective in reducing the harmful effects of Cd. It has been shown
8 that treatment of rats with Se prior to Cd administration prevented the effects of Cd, because
9 it bound Cd to low molecular weight, stable, and biologically inactive Cd selenide complexes
10 (Ohta et al. 1995). According to available data, it seems that the ability of Se to reduce Cd
11 accumulation in organs depends on Se/Cd ratio (Sasakura and Suzuki, 1998), their chemical
12 form and the solubility of the formed compounds (Feroci et al., 2005). Schrauzer (2009)
13 reported that the sequestration of toxic elements by Se represents an efficient detoxification
14 mechanism, but also results in the physiological inactivation and loss of Se. In our study, as
15 the age of broilers progressed, Se concentration in tissues decreased, possibly due to
16 involvement of Se in detoxification mechanisms. Decrease of tissue Se content support the
17 idea of Se-Cd complex formation but its excretion or deposition remains unclear. If Se
18 derivatives are supplemented as a remedy for heavy metal toxicity, they should form insoluble
19 compounds to be excreted. Indeed, early findings by Stowe (1976) have shown that Se
20 pretreatment increases the rate of biliary excretion of Cd.
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35 In the present study statistically significant positive correlations between Se - Cu and
36 Se - Zn were noted. Indeed, broilers fed Se unsupplemented diets exhibited reduced blood and
37 liver Zn levels as well as reduced liver Cu levels along with age. Similar results were reported
38 in several other studies where Se was positively correlated to Cu and Zn. In detail, it was
39 found that Cu was positively associated with Se in the liver (Lopez-Alonso et al. 2004) and in
40 the kidney (Blanco-Penedo et al. 2006) of cattle raised in Spain. It is possible that the Se - Cd
41 positive correlation may be related to the function of Se as a regulator of MT synthesis, which
42 may in turn hinder biliary excretion of Cu, leading to its accumulation. Earlier studies in
43 ruminants have also demonstrated that after injection of Se, Cu concentration in the liver
44 increased in animals receiving both normal (Hussein et al. 1985) or Cu deficient diets
45 (Thomson and Lawson 1970). However, Kotyzova et al. (2009) reported that in long term Se
46 deficiency of rats an increase of liver Cu concentration was noted. The positive correlation
47 between Se and Zn reported in the present study is in line with reports by Bou et al. (2005)
48 who noted that Zn supplementation (from 0 mg kg⁻¹ to 600 mg kg⁻¹) led to a significant
49 increase in Se content (115 to 213 µg kg⁻¹) in chicken meat. In the same study, in line with our
50 results, chicken muscle Zn content decreased with age. This positive Se - Zn correlation could
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3 be attributed to MT, the synthesis of which is induced by Zn and other cations (Nordberg
4 1998, Nordberg 2009).

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7 In the present study close negative correlations between Cd - Zn and Cd - Cu were
8 noted. These results are in line with previous studies by Elsenhans et al. (1987) where a
9 decrease of rat hepatic Cu concentration was observed after a 2-week administration of Cd.
10 Similarly, in the plasma of healthy humans, a negative association between Cd and Cu was
11 reported (Pasha et al. 2008). Cadmium shares physiological properties, such as MT-induction,
12 with other elements notably Cu and Zn having similar electron configurations in the outer
13 valence shell of the atom. These elements are thus often mutually antagonistic with exposure
14 to Cd lowering Cu status and exposure to Zn lowering Cd status (Underwood and Suttle
15 1999). Although it was initially thought that the only function of MTs was to provide a
16 defense against toxic cations (especially Cd), it is now known that these proteins have a
17 function in regulating normal Cu and Zn metabolism and in protecting cells against damage
18 induced by alkylating agents, oxygen radicals, and ionizing radiation (Lopez-Alonzo et al.
19 2002). Metallothioneins can also serve as store for Zn and Cd, and be involved in metal
20 transfer (Phillips et al. 2005; Sharma et al. 2005). The mechanisms by which Cd affects Zn
21 metabolism are not yet well understood but the strong association of Cd and Zn in the
22 examined tissues most likely is related to the fact that the handling of Cd²⁺ and Zn²⁺ in the
23 tissues intersect at several points. In our study, Cd concentration was greater in 6-wk old
24 broilers compared with its concentration in 4-wk old broilers. The observed Cd accumulation
25 with time is in line with observations by Nriagu et al. (2008) who reported that Cd contents of
26 older cows were much higher than those of younger animals. In other experimental models
27 (i.e. human or cattle) a positive association between Cd and Zn was noted. In detail, in plasma
28 of healthy humans (Pasha et al. 2008) and in the kidney of cattle (Lopez Alonso et al. 2002), a
29 strong positive correlation was observed between Cd and Zn, indicating that the organism and
30 possibly other factors like the mineral status may affect the association of Zn and Cd.
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35 Zinc was positively correlated with Cu in the present study. This result is in line with
36 findings by Nriagu et al. (2008) who reported a positive association of Cu and Zn in the liver
37 of free range cattle. Similarly, Blanco-Penedo et al. (2006) reported positive association
38 between Zn and Cu in the kidney of cattle raised in Spain. However, there are reports that
39 indicate that a negative correlation between Cu and Zn may exist. In detail, when dietary Zn
40 levels are very high, tissue Cu concentrations are very low and animals show clinical signs of
41 Cu deficiency or that low levels of Zn in the diet lead to Cu accumulation and Cu toxicity in
42 ruminants (Mills and Dalgarno 1972), pigs (Poulsen 1998) and laboratory animals (Blalock et
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3 al. 1988; Hall et al. 1979). Both Cu and Zn are integral parts of CuZn-SOD and both these
4 elements together with Se cooperate in the cytosolic antioxidant defense. Copper in excess of
5 cellular needs, mediates free radical production and direct oxidation of lipids, proteins and
6 DNA (Parge et al. 1992). Thus, it seems that a delicate balance between necessity and toxicity
7 for Cu and Zn exists which is achieved both at the cellular level and at the tissue and organ
8 levels (Valko et al. 2005).
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14 The present study revealed that in broilers, Fe was negatively correlated with Cu,
15 positively correlated with Se and uncorrelated with Cd or Zn. Several studies have confirmed
16 that Fe and Cu are associated (Gambling et al. 2008; Pasha et al. 2008). Copper -Fe
17 interactions may occur either at the level of intestinal absorption (Gambling et al. 2008) or
18 during transportation by the DMT1 (divalent metal transporter 1) (Garrick et al. 2003). Iron is
19 involved in the antioxidant defense system of the organism by the action of catalase which is
20 a tetrameric heme-containing enzyme, located primarily in the peroxisomes (Zoidis et al.
21 2010). Catalase detoxifies H₂O₂ by catalyzing its breakdown to water and divalent oxygen.
22 However, ferrous iron is able to trigger oxidations by reducing, as well as by decomposing,
23 previously formed H₂O₂ (Fraga and Oteiza 2002). The finding that Fe is correlated with Se, is
24 in line with previous reports (Uluozlu et al. 2009) and may indicate that occasionally there is
25 a necessity for antioxidant protection against Fe toxicity.
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36 In conclusion, trace elements may interact at the levels of absorption, distribution and
37 retention. Deficiency or supplementation of one of them may alter their balance and trigger
38 antagonistic or synergistic effects. The alteration of trace element balance may concomitantly
39 affect the antioxidant defense system since several trace elements, like Se, Cu, Fe and Zn, are
40 integral part of various antioxidant enzymes. The present study revealed that Se
41 supplementation, age of broiler and type of examined tissue affect the concentration of trace
42 elements. The level of supplemented Se is important since low or excessive quantities may
43 alter the balance and the correlation to the other elements. Although, significant trace element
44 correlations were reported in this study, they do not necessarily indicate that the level of
45 essential elements, like Se, can determine the amount of toxic metal accumulation in broiler
46 tissues or that toxic metal accumulation in tissues can affect essential element homeostasis.
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Table 1. Composition (g kg^{-1}) of control and Se-supplemented diets for chickens

Ingredients (g/kg)	Starter (0-2 wk)	Grower (2-4 wk)	Finisher (4-6 wk)
Maize	579.4	618.5	666.5
Soybean meal	332.8	287.2	251.5
Soya oil	42.7	52.7	43.0
Dicalcium phosphate	16.2	15.3	14.2
Limestone	13.6	11.1	10.8
Lysine	2.6	3.1	2.6
Methionine	3.7	3.8	3.4
NaCl	5.0	4.3	4.0
Premix ^a	4.0	4.0	4.0
Treatment	Se added ^b ($\mu\text{g kg}^{-1}$)	Se determined ^c ($\mu\text{g kg}^{-1}$)	
T1	0	210 \pm 14	
T2	150	443 \pm 19	
T3	300	685 \pm 29	
T4	3000	3683 \pm 210	

^a Premix supplied per kg of diet: 12000 IU vitamin A (retinyl acetate), 4000 IU vitamin D₃ (cholecalciferol), 80 mg vitamin E (DL- α -tocopheryl acetate), 9 mg vitamin K₃, 3 mg thiamin, 7 mg riboflavin, 6 mg vitamin B₆, 25 μg vitamin B₁₂, 50 mg nicotinic acid, 15 mg pantothenic acid, 1.5 mg folic acid, 0.15 mg biotin, 400 mg choline, 1.5 mg iodine, 50 mg iron, 130 mg manganese, 20 mg copper, 0.25 mg cobalt, 100 mg zinc. No Se was provided in the vitamin-mineral premix.

^b Se was added in the form of Sel-Plex (Alltech Inc, Nicholasville, KY, USA) and only in the Se-supplemented diets.

^c The determined Se content represents pooled data from all three diets (starter, grower and finisher).

Table 2. Determined and certified trace element concentrations in the reference material (NIST-RM 8414 -bovine muscle powder)

Element	Certified value ($\mu\text{g g}^{-1}$)	Detected Value ($\mu\text{g g}^{-1}$)	Recovery (%)
Se	0.076 ± 0.010	0.078 ± 0.011	103
Cd	0.013 ± 0.011	0.016 ± 0.002	123
Zn	142 ± 14	173 ± 1	122
Cu	2.84 ± 0.45	3.26 ± 0.08	115
Fe	71.2 ± 9.2	91.97 ± 1.03	129

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Table 3. The effect of Se supplementation in the diet of broilers on the concentration ($\mu\text{g kg}^{-1}$) of Se, Cd, Zn, Cu and Fe in blood, liver and breast tissue at 4th and 6th week of age

Factor Studied ^a	Trace Element				
	Se	Cd	Zn	Cu	Fe
Age (wk)					
4 th	876 ^a ±7.65	9.3 ^a ±0.37	12076 ^a ±208	1451±37.14	201635±7175
6 th	831 ^b ±7.35	16.4 ^b ±0.36	11454 ^b ±200	1354±35.68	196034±6893
Se added ($\mu\text{g kg}^{-1}$)					
0 (T1)	252 ^a ± 10.40	16.6 ^a ±0.50	11935±282	1452±50.46	180568± 9749
150 (T2)	441 ^b ± 10.40	9.8 ^b ±0.50	12031±282	1462±50.46	216623± 9749
300 (T3)	534 ^c ± 10.40	10.4 ^b ±0.50	11313±282	1340±50.46	202732± 9449
3000 (T4)	2186 ^d ± 11.20	14.5 ^c ±0.55	11781±305	1355±54.50	195416±10530
Tissue					
Blood	434 ^a ± 9.00	29.7 ^a ±0.44	3888 ^a ±244	393 ^a ±43.70	391203 ^a ±8442
Liver	1336 ^b ± 9.00	7.4 ^b ±0.44	25766 ^b ±244	3481 ^b ±43.70	199317 ^b ±8442
Breast	790 ^c ± 9.55	1.5 ^c ±0.46	5641 ^c ±259	332 ^a ±46.35	5985 ^c ±8955
Source of Variation	P Value				
Treatment (Se addition)	<0.001	<0.001	NS	NS	NS (P=0.078)
Age	<0.001	<0.001	0.034	NS (P=0.064)	NS
Tissue	<0.001	<0.001	<0.001	<0.001	<0.001
Treatment X Age	<0.001	<0.001	NS	NS	NS
Treatment X Tissue	<0.001	<0.001	NS	NS	<0.05
Age X Tissue	<0.001	<0.001	<0.01	NS	<0.001
Treatment X Age X Tissue	<0.001	<0.001	<0.05	<0.01	NS

^a The data are presented as means \pm SE (n = 4) of the main effect in turn, pooled for all other main effects, based on Multivariate Regression.

Means within a column, within a main effect comparison, with different superscripts are different at P< 0.05, unless otherwise stated.

Table 4. Correlations between the concentration of Se, Cd, Zn, Cu, Fe prior to and after the application of the statistical model

Prior to Model				
Application	Cd	Zn	Cu	Fe
Se	-0.213*	0.378***	0.356***	-0.112
Cd		-0.346***	-0.279**	0.654***
Zn			0.978***	-0.042
Cu				0.087
After the Model				
Application	Cd	Zn	Cu	Fe
Se	-0.389***	0.461***	0.312**	0.229*
Cd		-0.249*	-0.236*	-0.189
Zn			0.390***	0.073
Cu				-0.267*

Level of statistical significance of each of the pairwise correlations, * $P \leq 0.05$; ** $P \leq 0.01$;

*** $P \leq 0.001$.