AGRICULTURAL UNIVERSITY OF ATHENS

FACULTY OF ANIMAL SCIENCE AND AQUACULTURE

DEPARTMENT OF NUTRITIONAL PHYSIOLOGY AND FEEDING

Ph.D. Thesis

Διερεύνηση της επίδρασης προσθήκης Cd, Se και Zn στο σιτηρέσιο κρεοπαραγωγών ορνιθίων σε παραγωγικές και βιοχημικές παραμέτρους

Investigation of the effect of Cd, Se and Zn supplementation in broiler feed on productive and biochemical parameters

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To

My Heart and my soul (My Mother)

To

My sisters

And

My nephew Hussain

With all my love

ACKNOWLEDGMENTS

I would like to express my deepest thanks to my God (ALLAH)

My sincere gratitude and appreciation to the following persons and / or Institutions for their contribution to this thesis:

- Prof. K. Fegeros (Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding) for his support and his beneficial advises.

- Prof. G. Zervas (Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding) for his suggestions support and beneficial advises.

- Prof. I. Politis (Faculty of Animal Science and Aquaculture, Department of Animal Breeding and Husbandry) for his support, suggestions and beneficial advises.

- Dr. E. Zoidis (Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding) for his support, suggestions and beneficial advises.

- Dr. A.C. Pappas (Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding) for his support, suggestions and beneficial advises.

- IKY (State Schollarship Foundation).
- Dr. Nasser Nori Al- Anbari (Agriculture University of Baghdad - Iraq)
- Greek Embassy in Baghdad - Iraq
- Iraq Embassy in Athens - Greece
- Greek Embassy in NYC - USA
- Maria Georgiadou (Faculty of Animal Science and Aquaculture, Department of Nutritional Physiology and Feeding)

- Panagiotis Georgiou
- Tasia Pogthani
- Kathleen Covley
- Lisa Smith

Thank you all for your help.

Without you, this work would not have been completed.

AL-WAELI ALI

Contents

List of Tables

List of Pictures

Structure of the Thesis

The present thesis is divided in to three parts:

I. Part A: Introduction

II. Part B: Experiment 1

III. The role of selenium in cadmium toxicity: Interactions with essential and toxic elements

IV. The role of selenium in cadmium toxicity: Effects on broiler performance and health status

V. Part C: Experiment 2

VI. The effects of Zn, Se and Cd feed supplementation on broilers' performance, hematological parameters and accumulation of essential and toxic elements in various tissues

VII. Part D: General Discussion and conclusion

VIII. References

Περίληψη

Η πρόσληψη απαραίτητων ή τοξικών ιχνοστοιχείων από το περιβάλλον επιδρά σημαντικά στην ανάπτυξη των οργανισμών. Το σελήνιο (Se) είναι ένα απαραίτητο ιχνοστοιχείο που εμπλέκεται στη λειτουργία του αντιοξειδωτικού συστήματος των ζώων, ο ψευδάργυρος (Zn), είναι δομικό στοιχείο πολλών μεταλλοενζύμων ενώ το κάδμιο (Cd) ανήκει στα βαρέα μέταλλα, παρουσιάζει τοξική δράση και τα τελευταία χρόνια καταβάλλονται προσπάθειες περιορισμού της χρήσης και εναπόθεσής του στο περιβάλλον.

Σκοπός του πρώτου πειράματος της παρούσας μελέτης ήταν η χορήγηση Se και Cd στο σιτηρέσιο κρεοπαραγωγών ορνιθίων και η διερεύνηση των αλληλεπιδράσεών τους με άλλα ιχνοστοιχεία, της συσσώρευσής τους στους ιστούς, καθώς και των επιδράσεών τους στην φυσιολογική ανάπτυξη των ορνιθίων. Επίσης, στόχος ήταν να αξιολογηθεί εάν η προσθήκη αντιοξειδωτικών, όπως οργανικού σεληνίου (Se) και προ-οξειδωτικών, όπως καδμίου (Cd), μπορεί να επηρεάσει την συσσώρευση απαραίτητων και τοξικών στοιχείων (Se, Cd, Sb, Ca, Cr, Co, Cu, Fe, Pb, Mg, Mn, Mo, Ni, V και Zn) σε διάφορους ιστούς τους. Για τους σκοπούς του πρώτου πειράματος χρησιμοποιήθηκαν 300 νεοσσοί κρεοπαραγωγής (broilers) ηλικίας 1 ημέρας κατανεμημένοι τυχαία σε 4 επεμβάσεις (T1-T4) με 5 επαναλήψεις των 15 νεοσσών η κάθε μια. Στην επέμβαση T1 (μάρτυρας) χορηγήθηκε σιτηρέσιο με 0.3 ppm Se χωρίς την προσθήκη Cd, στις επεμβάσεις T2 και T3 χορηγήθηκε το σιτηρέσιο του μάρτυρα στο οποίο προστέθηκαν 10 ppm και 100 ppm Cd, αντίστοιχα, ενώ τέλος, στην επέμβαση T4 χορηγήθηκε σιτηρέσιο με προσθήκη 3 ppm Se και 100 ppm Cd. Το Cd στις επεμβάσεις T2, T3, T4 προστέθηκε ως $CdCl_2$, ενώ το Se χορηγήθηκε στην οργανική μορφή του (σεληνιο-αμινοξέα). Τα ορνίθια αναπτύχθηκαν για έξι εβδομάδες και ελήφθησαν δείγματα αίματος και ιστών (ήπατος, νεφρών και μυϊκού ιστού από 2 ορνίθια ανά κελί επανάληψης (συνολικά 40 ορνίθια) την $4^{η}$ και την $6^{η}$ εβδομάδα. Οι συγκεντρώσεις των στοιχείων προσδιορίστηκαν με τη μέθοδο της φασματοσκοπίας μάζας επαγωγικά συνδεδεμένου πλάσματος (ICP-MS). Μετρήθηκαν τα παραγωγικά χαρακτηριστικά και προσδιορίστηκαν αιματολογικά χαρακτηριστικά και βιοχημικοί παράμετροι.

Η επεξεργασία των παραγωγικών χαρακτηριστικών έδειξε ότι δεν υπήρχε στατιστικώς σημαντική διαφορά μεταξύ των επεμβάσεων T1 και T2 και μεταξύ των επεμβάσεων T3 και T4, αντίστοιχα, όσον αφορά στο τελικό σωματικό βάρος (g) ($2139^{a} \pm 60$, $2178^{a} \pm 80$, $950^{b} \pm 47$, και $1107^{b} \pm 50$). Τα ορνίθια που κατανάλωναν σιτηρέσια με 100 ppm Cd (T3 και T4),

παρουσίασαν υψηλότερο συντελεστή εκμετάλλευσης σε σχέση με αυτά των επεμβάσεων με καθόλου ή λίγο Cd (T1 και T2). Σημειώθηκε η τάση ο συντελεστής εκμετάλλευσης της επέμβασης T4 να είναι μικρότερος από αυτόν της T3, χωρίς όμως να είναι στατιστικώς σημαντική. Η θνησιμότητα δεν παρουσίασε στατιστικώς σημαντική διαφορά μεταξύ των επεμβάσεων. Επιπλέον, τα αιματολογικά και βιοχημικά χαρακτηριστικά δεν διέφεραν στατιστικώς σημαντικά και κυμαίνονταν εντός των φυσιολογικών τιμών.

Η ανάλυση των δεδομένων της ICP-MS έδειξε ότι ενώ το χαμηλό επίπεδο Cd στο σιτηρέσιο οδήγησε μόνο σε αύξηση της συγκέντρωσης Cd στους εξεταζόμενους ιστούς, τα υψηλά επίπεδα Cd είχαν ως αποτέλεσμα αύξηση των συγκεντρώσεων των Cd, Cu, Sb και V και μείωση των συγκεντρώσεων των Se, Mn και Fe. Η προσθήκη υψηλών επιπέδων Se δεν μείωσε στατιστικώς σημαντικά τη συγκέντρωση Cd. Η συγκέντρωση του Cd αυξήθηκε με την ηλικία των ορνιθίων, κάτι που δεν παρατηρήθηκε για το Se, τον Cu και τον Zn. Η επιπλέον προσθήκη Se στο σιτηρέσιο που είχε ήδη 100 ppm Cd (T4 έναντι T3), αύξησε μεν τη συγκέντρωση του Se στους ιστούς ($P<0.001$), αλλά δεν μείωσε στατιστικώς σημαντικά τη συγκέντρωση του Cd, ούτε επηρέασε αυτές των Cu και Zn. Οι νεφροί παρουσίασαν τη μεγαλύτερη συσσώρευση ιχνοστοιχείων ακολουθούμενοι από το ήπαρ, το μυϊκό ιστό και το αίμα. Μετά από εφαρμογή κατάλληλου στατιστικού μοντέλου παρατηρήθηκαν περισσότερες από 39 συσχετίσεις μεταξύ των συγκεντρώσεων των εξεταζόμενων στοιχείων. Οι πιο σημαντικές αφορούσαν στη συσχέτιση του Cd με τα Ca, Co, Cu και Mg, ενώ το Se συσχετίστηκε με το Mn.

Η παρούσα μελέτη επιβεβαίωσε την αρνητική επίδραση των υψηλών συγκεντρώσεων του Cd στην ανάπτυξη των πτηνών. Η θετική συσχέτιση του Cu και του Zn με το Cd, υποδηλώνει την ανάγκη του οργανισμού να αυξήσει τη συγκέντρωση των ιχνοστοιχείων με προστατευτική δράση παράλληλα με την αύξηση της συγκέντρωσης του τοξικού Cd. Υπό αυτή την έννοια, η προσθήκη υψηλής συγκέντρωσης Se είχε την τάση να μετριάσει τις αρνητικές επιδράσεις του Cd. Τα αποτελέσματα της μελέτης υποδηλώνουν την λεπτή ισορροπία μεταξύ ιχνοστοιχείων με τοξική και αντιοξειδωτική δράση και πως αυτή μεταβάλλεται όταν η συγκέντρωση ιχνοστοιχείων με τοξική δράση αυξηθεί. Τέλος, τα αποτελέσματα του πρώτου πειράματος αποκάλυψαν αρκετές συσχετίσεις μεταξύ των απαραίτητων, πιθανώς απαραίτητων και τοξικών στοιχείων καταδεικνύοντας τη σημασία της ισορροπίας μεταξύ των προ-οξειδωτικών και των αντιοξειδωτικών.

Σκοπός του δεύτερου πειράματος της παρούσας μελέτης ήταν η διερεύνηση της επίδρασης χορήγησης των μέγιστων επιτρεπτών συγκεντρώσεων Se και Zn στο σιτηρέσιο κρεοπαραγωγών ορνιθίων που είχαν επιμολυνθεί με 50 ppm Cd επί των παραγωγικών τους χαρακτηριστικών, επί της συσσώρευσης δεκαεπτά απαραίτητων και τοξικών στοιχείων σε διάφορους ιστούς καθώς και η διερεύνηση των αλληλεπιδράσεων των παραπάνω στοιχείων.

Για τους σκοπούς του δεύτερου πειράματος χρησιμοποιήθηκαν 180 νεοσσοί κρεοπαραγωγής (broilers) ηλικίας 1 ημέρας κατανεμημένοι τυχαία σε 3 επεμβάσεις (T1-T3) με 4 επαναλήψεις των 15 νεοσσών η κάθε μια. Στην επέμβαση T1 (μάρτυρας) χορηγήθηκε σιτηρέσιο με 0.3 ppm Se (σεληνιο-αμινοξέα) και 100 ppm Zn (ZnO) χωρίς την προσθήκη Cd. Στην επέμβαση T2, χορηγήθηκε σιτηρέσιο με 0.3 ppm Se, 150 ppm Zn και προσθήκη 50 ppm Cd ($CdCl_2$). Ενώ τέλος, στην επέμβαση T3 χορηγήθηκε σιτηρέσιο με προσθήκη 0,5 ppm Se, 150 ppm Zn και 50 ppm Cd. Τα ορνίθια αναπτύχθηκαν για έξι εβδομάδες και ελήφθησαν δείγματα αίματος και ιστών (ήπατος, νεφρών, μυϊκού ιστού) από 2 ορνίθια ανά κελί επανάληψης την $6^{η}$ εβδομάδα. Μετρήθηκαν τα παραγωγικά χαρακτηριστικά και προσδιορίστηκαν αιματολογικά χαρακτηριστικά και βιοχημικοί παράμετροι. Οι συγκεντρώσεις των στοιχείων προσδιορίστηκαν με τη μέθοδο της φασματοσκοπίας μάζας επαγωγικά συνδεδεμένου πλάσματος (ICP-MS).

Η προσθήκη 50 ppm Cd στο σιτηρέσιο κρεοπαραγωγών ορνιθίων οδήγησε σε μείωση των επιδόσεων τους, η οποία δεν βελτιώθηκε σημαντικά με την προσθήκη Se και Zn. Παρ 'όλα αυτά, οι αιματολογικές παράμετροι που εξετάστηκαν κυμάνθηκαν εντός των φυσιολογικών τιμών και δεν αποκαλύπτουν ιδιαίτερα αρνητικές επιπτώσεις στην υγεία μετά από ταυτόχρονη προσθήκη Cd, Se και Zn. Η παρούσα μελέτη έδειξε ότι τα απαραίτητα ιχνοστοιχεία Se και Zn μπορούν να συμβάλλουν εν μέρει στη βελτίωση από τις αρνητικές επιπτώσεις του Cd, τουλάχιστον στα επίπεδα συσσώρευσης του Cd στους ιστούς, αλλά δεν μπορούν να αντισταθμίσουν όλες τις αρνητικές επιπτώσεις του. Επιπλέον, η μελέτη αποκάλυψε αρκετές συσχετίσεις μεταξύ απαραίτητων, πιθανώς απαραίτητων και τοξικών στοιχείων που απεικονίζουν τη σημασία της ισορροπίας μεταξύ των προ-οξειδωτικών και αντιοξειδωτικών.

Abstract

The intake of essential or toxic trace elements from the environment plays a significant role in the growth of organisms. Selenium (Se) is an essential element involved in the antioxidant system of animals and zinc (Zn) is a constituent of prosthetic groups in metalloproteins and metalloenzymes while cadmium (Cd) is identified as a heavy metal that causes toxic effects leading the last years to an effort of minimizing its usage and storage to the environment.

The aim of the first experiment was to investigate if organic Se can ameliorate the toxic effects of Cd. We investigated if Se and Cd addition to chickens' diet affect the accumulation of Se Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn in the tissues. Three hundred one day-old, chickens (broilers) were randomly distributed in four dietary treatments with 5 replicate pens per treatment. In treatment T1, chickens were fed a diet with 0.3 ppm added Se, as Se yeast, without added Cd. In T2, chickens were fed a diet with 0.3 ppm Se and 10 ppm Cd, as $CdCl_2$. In T3, chickens were fed a diet with 0.3 ppm Se and 100 ppm of Cd and in T4 chickens were fed a diet with 3 ppm Se and 100 ppm Cd. On days 28 and 42, two chickens per replicate pen were sacrificed for collection of whole blood, liver, kidney and breast muscle samples. Determined were productive characteristics and hematological and biochemical parameters. The elements' concentrations were analyzed by inductive coupled plasma- mass spectrometry (ICP-MS).

Statistical analysis of the performance characteristics showed that concerning the body weight (g), there was no statistically significant difference between T1 and T2 as well as between T3 and T4 treatments ($2139^{a} \pm 60$, $2178^{a} \pm 80$, $950^{b} \pm 47$ and $1107^{b} \pm 50$). Broilers consuming feed with 100 ppm Cd (T3 and T4), showed higher feed consumption ratio (FCR) compared to treatments without Cd (T1) or 10 ppm Cd (T2). FCR in T4 tended to be lower than in T3 while mortality showed no statistically significant differences between all treatments.

The ICP-MS data analysis showed that while the low Cd level (10 ppm) in feed led to high concentrations of Cd in the examined tissues, high Cd levels led to increased concentrations of Cd, Cu, Sb and V and decreased concentrations of Se, Mn and Fe. The addition of Se at high levels did not reduce Cd concentration. Cd levels rose with the age of broilers, a fact that did not occur for Se, Cu and Zn. The extra addition of Se to the feed containing 100 ppm Cd (T4 vs T3)

raised the concentration of Se in the tissues ($P<0.001$), but did not reduced Cd levels nor affected Cu and Zn concentrations. Kidneys showed the greatest element accumulation followed by liver, breast muscles and blood. After applying the proper statistical model, more than 39 correlations between the examined trace elements were noticed. Most significant ones were noted between Cd with Ca, Co, Cu, and Mg while Se was correlated only with Mn.

The present study confirmed the negative effects of high Cd concentrations on broiler development. The positive correlation of Cd with Zn and Cu indicates the need of the organism to accumulate elements with protective action as a countermeasure for increased Cd. The results of the study indicate that there is a thin balance between toxic elements and elements with anti-oxidant action and the way this balance is modified by high levels of toxic elements. Finally, the results of the first experiment revealed several correlations between essential, potentially essential and toxic elements indicating the importance of equilibrium between the pro-oxidative and anti-oxidative states.

The aim of the second experiment was to assess if organic selenium (Se) and Zn can protect against the toxic effects of cadmium (Cd) and to examine if Se, Zn and Cd addition to chickens' diet affect the accumulation of Se Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn in the tissues.

A total of 180 one day-old, as hatched, broilers were randomly distributed in three dietary treatments with 4 replicate pens per treatment. In T1 treatment, chickens were fed a diet with 0.3 ppm added Se (as Se yeast), 100 ppm Zn (as ZnO), without added Cd. In T2, chickens were fed a diet with 0.3 ppm Se, 150 ppm Zn and 50 ppm Cd (as $CdCl_2$). In T3, chickens were fed a diet with 0.5 ppm Se, 150 ppm Zn and 50 ppm Cd. On the 6^{th} week, three broilers per replicate pen were sacrificed for the collection of whole blood-, liver-, kidney- and breast samples. Body mass, feed conversion ratio and mortality were assessed and hematological analyses were performed. Selenium, Zn and Cd levels, as well as the levels of fourteen additional essential and toxic elements in the selected tissues were analyzed by ICP-MS.

Addition of 50 ppm Cd to broilers' diets had negative effects on their performance which was not significantly improved by the addition of Se and Zn. Nevertheless, the examined hematological parameters ranged within physiological values revealing no negative health effects after simultaneous Cd, Se and Zn addition.

The present study indicated that Se and Zn can partially compensate for the negative effects of Cd, at least at the levels of Cd tissue accumulation, but cannot counteract all of its negative effects. In addition, the study demonstrated several correlations between essential, probably essential and toxic metals reflecting the importance of the balance between pro-oxidants and antioxidants.

Part A

BACKGROUND

1.1. Trace elements

Micronutrients, called also trace elements or trace minerals, have an essential role in all kind of species: crops, livestock, and humans. They include those nutrients required in extremely small quantities by the organism. Their lack, e.g., in crops and livestock can cause serious crop production or animal health problems (Mengel, 1980; Miller et al., 1991). Serving as constituents of prosthetic groups in metalloproteins and as activators of enzyme reactions they function most importantly as a “spark plug”, without which the enzyme system in living beings would simply be an inert mass of proteins. The most efficient means of exposure to metals is obviously considered to be the ingestion of food, as many metals are natural components of foodstuffs, not forgetting environmental contamination and contamination during processing.

Both developed and undeveloped countries continue to face daily environmental pollution (Atta et al., 1997). Torra et al. (2002) reported that heavy metal contamination was the immediate result of rapid industrialization in every developing country, due to lack of environmental control. All the natural forms of the elements are usually found in relatively low concentrations in the ecosystem, nevertheless anthropogenic activities influence also their presence concerning mostly their quantity.

Major pollutants, such as waste burning, fertilizer, vehicle emissions, agricultural and sewage sludge dispersed by man-made emission sources have provoked an important increase in the overall concentration of elements in the environment because of the intensity and the persistence of all anthropogenic sources in the recent decade (Ansari et al., 2009; Jamali et al., 2008; Saxena, et al., 2008). As anticipated, different parts of the world revealed different levels of contamination, derived from metalliferous mining, and consequently resulted in very high levels of heavy metals in areas of agricultural lands, affecting the plants growing on the polluted soils and grazing animals which exhibited increased concentrations of heavy metals (Kelepertsis and Bibou, 1991).

Burning of coal and lignite (brown or soft coal) and use of leaded gasoline, apart from mining and refining, remain a major source of pollution with heavy metals. Interaction between metals or metalloids is the main reason for a severe modulation as far as toxicity is concerned, not that relatively high concentrations can’t also occur naturally (WHO, 1996; Goyer, 1995). The heavy metals can be transferred from soil to animals either by direct contamination or via

vegetation. Even though soil as a biochemical reactor is an active filter for trace metals, these may accumulate in the vegetation and, thus, create a risk for animals and humans (Sedki, et al., 2003; Hogue, et al., 1984). In higher organisms, intake of trace elements pass mainly through the respiratory systems or through the food chain, in fact many dangerous elements or compounds, such as dioxins, pesticides, metals, and metalloids, accumulate along the food chain (Ghaedi et al., 2008; 2007; Prankel et al., 2004; Alberti-Fidanza et al., 2003; Toso et al., 2002).

Trace elements can be classified in three distinct categories: potentially toxic (e.g. cadmium, arsenic, aluminum, lead, mercury), probably essential (e.g. nickel, vanadium and cobalt) and essential (e.g. selenium, manganese, copper, zinc and iron) (Jalbani et al., 2007; Munoz-Olivas et al., 2001). The essential metals can produce toxic effects when the metal intake is excessively elevated (Celik et al., 2007; Pouretedal et al., 2007) but it's the nonessential metals (e.g., Pb, Cd, and Hg) that have proven to be the most dangerous, since continuous exposure of organisms to their low concentrations may result in bioaccumulation and subsequent transfer to people by the food chain (Chale, 2002; Kristl et al., 2003). Pb and Cd are not natural substances in nutrition. Essential micronutrients such as Se, Zn, Cu, Mn, Fe, Co and Mo are required in amounts ≤ 100 mg/kg^{-1} in dry matter which with the exception of Mo is deficient in some natural feed ingredients, necessitating their supplementation.

Both industrial and agricultural activity has shown increased environmental concentrations of trace metals, such as Cu and Zn, in certain areas. Essential elements as these, of which deficient intake ends up in impairment of biological functions are toxic when ingested in excess (Friberg et al., 1979). The ingestion of a diet that is lacking a particular essential element can enhance the accumulation and toxicity of some toxic metals. Calcium, Fe and Zn deficiency, for instance, enhance susceptibility to Cd and Pb toxicity (Goyer, 1997; WHO, 1996).

The presence of trace elements in farm animals is of interest from both the animal health and human health perspectives (Nriagu, 1986; Bowen, 1978). Exposing livestock to either high levels of toxic metals (such as Cd and Pb) or less than optimal levels of the essential microelements (such as Cu, Co and Zn) can engender adverse effects such as reproductive impairment, physiological abnormalities, behavioral modifications or even death (Sharma et al., 2005; Custer et al., 2004; Martelli and Moulis, 2004; Tapiero and Tew, 2003; Dorton et al., 2003; Frank et al., 2000). Metals accumulated in livestock can be passed on to people who

consume the meat and constitute a health hazard. Of all the animal tissues, kidney and liver raise a special dilemma since they have a propinquity to bioaccumulation of toxic metals such as Pb, Cd, Hg and As (Alonso et al., 2002; 2000) but can also serve as a rich source of essential microelements (notably Fe, Cu, Zn and Se) in human diet (Arnold et al., 2006; Vos et al., 1987).

1.2. Cadmium

1.2.1. Identity and origin

Cadmium has atomic number 48 and belongs in the IIB group (metals) on the periodic table. It is a nonessential soft, silver-white metal that is found naturally at low levels in rocks and soil (Research Triangle Institute et al., 1999). Cadmium is used in a variety of industries, e.g., in nickel–cadmium batteries, electroplating, as a component in metallurgical and brazing-soldering alloys, in pigments, and as a stabilizer for plastic. Most of the cadmium is released in the environment via the smelting of other metals, notably, zinc. Other sources of environmental cadmium are the burning of fossil fuels and waste materials (often deposited as solid waste), and the use of high phosphate and sewage sludge fertilizers (IARC, 1993).

Approximately 4,000 to 13,000 tons of cadmium is released into the environment from human activities annually (Research Triangle Institute et al., 1999. Industrial emissions and fertilization have caused a significant rise in cadmium levels in arable soils and crops during the 20^{th} century (Satarug et al., 2003). Cadmium in soil is taken up selectively by edible plants, multiplying concentrations of cadmium compared to those of the surrounding soil (Satarug and Moore, 2004; Satarug et al., 2003).

Furthermore, Cd is present as a contaminant in food (leafy vegetables, grains, and cereals), water, and tobacco leaves, as well as byproduct of zinc and lead mining and smelting. Because of its widespread nature, cadmium can either be ingested via contaminated foods or inhaled. Even in small amounts (100-200 ppm) its effects are not to be taken lightly as it affects the kidneys and the gastrointestinal tract in mammals (Bremner, 1978). Other manifestations of cadmium toxicity include mild anemia and osteoporosis although the most pronounced effects occur in the kidney. When significant cadmium damage such as proteinuria and decreased renal

function has taken place then significant cadmium excretion occurs (Hayter, 1980). The most dangerous characteristic of cadmium is that it accumulates throughout a lifetime.

Cadmium is a nonessential ubiquitous heavy metal which has been categorized as "category I" human inhalational carcinogen (IARC, 1993; FAO/WHO, 1999), a mutagen and a teratogon. Numerous studies on cadmium toxicity have been undertaken, and in mammals the target organs for accumulation (due to organotropism) after chronic low-dose exposure are primarily kidney and liver (Prankel et al., 2005). Cadmium accumulated in the liver and kidney has a long biological half-life of 17 to 30 years in humans. In fact, it has been claimed that approximately one-third of the cadmium accumulated in the body of an adult was absorbed during the first few years of his life (Henke et al., 1970).

The concentration of cadmium in pig kidneys (a surrogate marker for human exposure) has increased at a rate of ~2% per year in the period of 1984-1992 (Grawè et al., 1997). Estimates of dietary cadmium intake worldwide range from 10 to 40 μg/day in non-polluted areas to several hundreds of micrograms in cadmium-polluted regions (Järup et al., 1998). Moreover, seafood, organ meat (liver and kidney), potatoes, and root vegetables can contain high levels of cadmium (Satarug et al., 2003; Research Triangle Institute, et al. 1999). Cadmium has been shown to interact with the metabolism of four metals essential to nutrition: zinc, iron, calcium and copper (Goyer, 1995; Bremner, 1978; Petering, 1978).

1.2.2. Cadmium metabolism

Cadmium metabolism has several unique characteristics (Goering et al., 1994; Klaassen et al., 1981) and its absorption shows marked route dependency. Only about 5% of a given dose of cadmium is absorbed from the gastrointestinal tract while cadmium absorption from the lung is very high, with as much as 90% of a dose deposited in the deep lung being absorbed. Once absorbed, cadmium is rapidly cleared from the blood and concentrates in various tissues. Cadmium in the liver and kidney usually make up the bulk of the total body burden (Goering et al., 1994; Klaassen et al., 1999). Hepatic and renal accumulation may be due to the ability of these organs to produce large amounts of metallothionein (MT).

Metallothionein is a low molecular weight cysteine-rich, intracellular protein with high affinity for both essential and non-essential metals (Klaassen et al., 1999). When Cd concentration exceeds the binding capacity of MT, the non-bound Cd causes toxicity possibly due to free radical induction and lipid peroxidation (Sarkar, et al. 1997). The toxic effects of cadmium often stem from interference with various zinc mediated metabolic processes, and zinc treatments frequently reduce or abolish the adverse effects of cadmium (Goering, et al. 1994).

Cadmium disrupts the antioxidant enzyme system, causes enzyme dysfunction, resulting in increased oxygen radical production, which causes damage to membranous structures such as mitochondria and endoplasmic reticulum (Schrauzer, 2009; Ognjanovic et al., 2010). Cadmium is known to induce the production of nitric oxide (NO) and reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions radicals and H_2O_2.

1.2.3. Cadmium carcinogenesis

Cadmium is a heavy metal, considered contaminant; it has been reviewed by the International Register of Potentially Toxic Chemicals of the United Nations Environment Programmed and included on the list of chemical substances and processes as potentially dangerous at global level (IRPTC, 1987). It is toxic, teratogenic, mutagenic and carcinogenic to most organisms (Robards and Worsfold, 1991). Commercial cadmium production started early last century, but the pattern of cadmium consumption has changed in recent years with significant decreases in electroplating and increases in nickel-cadmium batteries and specialized electronic uses (Järup, 2003; IPCS, 1992; Robards and Worsfold, 1991).

The earliest suspicion that cadmium might be carcinogenic in rodents came from a 1961 study by Haddow et al. (1961) who gave either subcutaneous (sc) or intramuscular (im) injections of ferritin which had been prepared from rat liver by cadmium precipitation. They subsequently found malignant tumors at the site of injection in rats and mice, though, it was unclear at that time if cadmium was the carcinogenic agent, but it was suspected (Haddow et al., 1961).

These results prompted further investigations and cadmium now has been established as a potent rodent carcinogen for over 35 years. The carcinogenic potential of cadmium was first shown at repository type injection sites, such as im or sc, where it forms sarcomas at high incidence (Waalkes et al., 1996; IARC, 1993). Early studies also showed cadmium to be an effective testicular tumorigen with a single exposure producing a high incidence of testicular interstitial cell tumors (Waalkes et al., 1995; 1996; IARC, 1993).

1.2.4. Cadmium in different tissues

Acute inhalation exposure to cadmium at concentrations above 5 mg m^{-3} may cause destruction of lung epithelial cells, resulting in pulmonary edema, tracheobronchitis, and pneumonitis. The respiratory response to cadmium is similar to the response seen with other agents that produce oxidative damage. Typically, it occurred is an alveolar pneumocyte type 2 cell hyperplasia in response to type 1 cell damage and necrosis. Long-term inhalation exposure at lower levels also leads to decreased lung function and emphysema. Some tolerance to cadmium-induced lung irritation develops in exposed humans, and respiratory function may recover after cessation of cadmium exposure (IARC, 1993).

Another effect of long-term cadmium inhalation exposure is damage of the olfactory function. Nonoccupational exposure to cadmium is unlikely to be high enough to cause significant respiratory effects (Leduc et al., 1993; Sorahan and Lancashire, 1997). Conflicting evidence has been obtained for the effect of cadmium exposure on the cardiovascular system. In some studies on rats, rabbits, and monkeys, cadmium exposure was shown to increase blood pressure, or to cause cardiac lesions. However, studies of exposed humans have found positive, negative and no association between cadmium exposure and hypertension. This suggests that if cadmium does affect blood pressure, the magnitude of the effect is small compared to other determinants of hypertension. Death rates for cardiovascular disease do not appear to be elevated in populations exposed to cadmium by inhalation or the food chain.

Overall, the weight of evidence suggests that cardiovascular effects are not a sensitive end point indicator for cadmium toxicity (Kopp, 1982; Steassen and Lauwerys, 1993). The

gastrointestinal tract is the target organ for high-level, acute, oral exposure to cadmium in both humans and animals, due to direct irritation of the gastric epithelium. The main symptoms following ingestion of cadmium at doses above 0.07 mg kg^{-1} in humans are nausea, vomiting, and abdominal pain. Gastrointestinal toxicity is not observed after lower levels of oral exposure or after inhalation exposure to cadmium, indicating that gastrointestinal effects are not likely to occur from environmental exposures to cadmium (Shipman, 1986). Both oral and inhalation exposure to cadmium can cause anemia. Oral exposure to cadmium has been shown to reduce uptake of iron from the diet in animals. It is likely that cadmium transported to the gastrointestinal system from the lung following inhalation exposure would also reduce iron absorption. Therefore, anemia induced by inhalation exposure to cadmium is likely to be caused by reduced iron absorption (Friberg et al., 1985). Prolonged inhalation or ingestion exposure of humans to cadmium causing renal dysfunction can lead to painful and debilitating bone disease in individuals with risk factors such as poor nutrition. Evidence from human studies suggests that low-level chronic exposure to cadmium causes alternations in renal metabolism of vitamin D, which then may cause milder bone effects such as osteoporosis.

These effects may be compounded by loss of calcium and phosphate with more severe renal damage, leading to osteomalacia (softening of the bones caused by defective bone mineralization). A recent large-scale cohort study in Belgium found that increased urinary calcium excretion was significantly associated with urinary cadmium levels, an index of kidney cadmium burden. This evidence suggests that either cadmium may have a direct effect on bone at levels lower than those causing kidney damage, or that interference with vitamin D metabolism in the proximal tubule may be a more sensitive indicator of cadmium-induced renal damage than proteinuria (Jarup et al., 1998; Steassen et al., 1999).

The kidney is the main target organ for cadmium toxicity following intermediate- or chronic-duration exposure by the inhalation or oral routes, as has been shown by numerous studies. The first manifestation of kidney damage is decreased reabsorption of filtered low molecular-weight proteins, indicating damage to the renal tubules. Production of tubular proteinuria is a relatively specific effect of cadmium on the kidneys and has been observed even following acute parenteral exposure in animals (Waalkes and Goering, 1990). This damage has been associated with increased urinary levels of β2-microglobulin, retinol-binding protein, or other low-molecular-weight proteins. At higher levels or durations of exposure, increased

excretion of high molecular-weight proteins occurs, indicating either glomerular damage or severe tubular damage. The sensitivity of the kidney to cadmium is related to the metabolism of cadmium in the body. Notably, except for extremely high-dose exposure, cadmium exists in the body primarily bound to metallothionein.

The Cd-metallothionein complex is readily filtered at the glomerulus and reabsorbed in the proximal tubule. Within the tubular cells, the metallothionein is degraded in lysosomes and free cadmium is released. The synthesis of endogenous metallothionein by the tubular cells is then stimulated, but when the total cadmium content in the renal cortex exceeds approximately 200 μg g^{-1} wet weight, the amount of cadmium not bound to metallothionein becomes sufficiently high to cause tubular damage (Waalkes and Goering, 1990; Buchet et al., 1999) The health significance of the early kidney damage is difficult to assess. The decreased resorption of low molecular-weight proteins is not adverse in and of itself, but may be indicative of increased excretion of other solutes. Deaths from renal failure due to cadmium exposure are rare, but even after cadmium exposure ceases, the renal damage continues to progress. Evidence that cadmium exposure may affect kidney vitamin D metabolism with subsequent disturbances in calcium balance and bone density suggests that decreased bone density, particularly in elderly women, may be a significant adverse effect of kidney cadmium accumulation (WHO, 1992a; WHO, 1992b; ATSDR, 1999).

1.2.5. Cadmium in chicken

Cadmium (Cd) is an environmental pollutant ranked eighth in the Top 20 Hazardous Substances Priority List (ATSDR, 1999). With a maximum tolerable level of 0.5 ppm (NRC, 1980), cadmium can be considered the most toxic of the heavy metals. However, that value was chosen based more on the safety of carryover to the human food supply as opposed to signs of toxicosis observed in the animals consuming the element. Severe effects have been reported in animals consuming 5 ppm in the diet or 1 ppm in drinking water with adverse effects occurring at dietary levels as low as 1 ppm. The most likely source of contamination in the animal feed industry is in conjunction with the use of zinc sulfate or poorly processed zinc ores as sources of

supplemental zinc. Other potential sources include mining and smelting operations, corrosion of metal-plated iron, discarded cadmium-chloride products, and the use of urban sewage sludges to fertilize pastures or croplands (NRC, 1980). Cadmium shares similarities in chemical reactivity with zinc and these two elements have common metabolic pathways in biological systems. The cadmium content of practical-type diets for poultry generally contain from about 0.05 to 0.35 ppm (NRC, 1980).

Induction of synthesis of metallothionein in the intestinal tract is the primary protective mechanism the animal has to prevent absorption of toxic amounts of cadmium. The element is sequestered by the protein and the epithelial tissue subsequently sloughed and eliminated in faeces. When Japanese quail were fed a diet containing 1 ppm radio-cadmium for 1 week, followed by 50 days on a basal diet, less than 4% of the initial dose remained (Jacobs et al., 1997). Approximately 25% was found in liver and another 25% in kidneys with 12% in the intestinal tract, probably in conjunction with metallothionein. Cadmium which is absorbed into tissues is retained by the body for a considerable period of time, thus exposure should be minimized, especially in breeders and layers as opposed to broilers or turkeys. Kidney is the primary site of damage from the element. Addition of dietary ascorbic acid and selenium had the greatest protective effects on broiler kidney damage from cadmium consumption (Rambeck and Kollmer, 1990).

1.2.6. Cadmium bound to metallothionein

After absorption, Cd circulates in erythrocytes or it is transported linked to albumin into the liver where it is taken by hepatocytes. It can induce and link to metallothioneins (MT) in the liver, which are, as mentioned earlier, cysteine-rich proteins that can concentrate Cd up to 3000 times (Klaassen et al. 1999; Nordberg, 2004). Due to numerous cysteines with thiol groups (–SH), MT have a high affinity for various reactions with metals. MT expression is induced by a metal-responsive transcriptional factor 1 (MTF-1) as a response to various stresses via linking with metal-responsive element located in promoting regions of target genes (Bi et al., 2004) that contain two cysteines and two histidines (Giedrocet al., 2001), and in this way, it represents a

potential target for cadmium intoxication. Both events, the reduction of MT expression and glutathione (an important antioxidant tripeptide) level, increase cellular damage due to cadmium exposure. The existence of cadmium-binding protein (MT) has been known for quite some time, not only in the kidneys, liver, and pancreas but also in the thyroid gland in human (Sato and Takizawa, 1982). At the same time, these organs displayed an affinity to accumulate high levels of cadmium.

Cadmium is not biodegradable and is redox inert when compared to other metals (Fe and Cu). Little is known about Cd's biotransformation except its conjugation with sulfhydryl groups such as MT and glutathione (Klaassen et al., 1999; Nordberg, 2004). Molecules that are different from MT, such as albumin, cysteine, glutathione, and proteins rich in sulfhydryl groups, can also form compounds with Cd (Wimmeret al., 2005). Due to the capability of binding Cd, MT is Cd's efficient intracellular detoxifier. The protective role of MT in cadmium toxicity is confirmed not only with acute Cd poisoning but also with chronic Cd toxicity and Cd carcinogenesis (Klaassen et al., 2009). It is well known that MTs modulate three fundamental processes: releasing gas mediators such as hydroxyl radical or nitric oxide, apoptosis, and the binding and exchange of heavy metals such as Zn, Cd, and copper (Thirumoorthy et al., 2011).

The presence of MT in all mammal cell types indicates that these MT play an important role in intracellular functions, but it is thought that their role is not vital for survival and reproduction (Vasák and Hasler, 2000). In mammals' cells, MT is located in certain compartments, for instance, cytoplasm, lysosomes, mitochondria, and the nucleus, which indicates a compartmentalized function in health and disease. Many studies have indicated the leading role of cytoplasmic MT in the homeostasis of Zn and Cu (Nordberg & Nordberg, 2009; Petering, Krezoski and Tabatabai, 2009; Tapia et al., 2004; Vasák and Hasler, 2000) and in the protection of or decrease in oxidative stress induced with toxic metals and other states (Klaassen et al., 2009; Liu et al., 2009; Petering et al., 2009).

1.3. Selenium

1.3.1. Identity and origin

Selenium (Se) is a chemical element with atomic number 34 belonging to the VI group of the periodic table of elements. In nature Se exists in two chemical forms, organic and inorganic. Elemental Se can be reduced to Se^{-2} oxidation state (selenide) or oxidized to the Se^{+4} (SO_3^{-2}, selenite) or Se^{+6} (SO_4^{-2}, selenate). Therefore, inorganic Se can be found in different minerals in the form of selenite, selenate and selenide as well as in the metallic form. In contrast, in feed ingredients (forages, grains, oilseeds etc) Se is an integral part of the amino acids methionine and cysteine and exists in the Se^{-2} oxidation state. As a result, in nature animals receive Se mainly in the form of selenomethionine (Se-Met) (Combs, 1986). The plant absorbs Se from the soil in the form of selenite or selenate and synthesizes seleno-amino acids with Se-Met representing about 50% of the Se in cereal grains (Olson and Palmer, 1976) with Se-methyl-selenomethionine, selenocysteine and Se-methyl-selenocysteine being the other seleno-compounds found in plants (Brody, 1994).

The release of Se compounds has been hastened by industrial and agricultural activity, making them available to fish and wildlife in aquatic and terrestrial ecosystems around the globe. Recent investigations on contaminant Se concluded that Se exhibits its toxicity in animals primarily through the food chain (Hamilton, 2004; Forest et al., 1999; Lemly, 1999). Furthermore, agricultural drain water, sewage sludge, fly ash from coal fired power plants, oil refineries and mining of phosphates and metal ores are all sources of Se contamination in the aquatic environment. Specifically, bivalves (being filter-feeders) have been identified as the most sensitive indicators of Se contamination (Hamilton, 2004). It was made obvious that accumulation of Se in marine animals from dietary sources (phytoplankton and zooplankton) surpasses significantly that accumulated directly from the water. In addition, Se compounds are widely used in glass manufacture, electronic applications, photocopy machines, inorganic pigments, rubbers, ceramics, plastics and lubricants (Akl et al., 2006). Food processing such as cooking (boiling, baking or grilling) could deescalate Se food content by volatilization (Dumont et al., 2006; Sager, 2006).

In plants, Se appears as part of Se-Met (Schrauzer, 2003). Selenium content in foods and beverages varies geographically and it is also reflected through its levels noted in animal products and plants. It depends on their consumed diet when it comes to animals (Barclay et al., 1995) and the soil in which the plants are grown. Se in the form of selenate or selenite is taken up by plants and mainly transformed into Se-Met in cereal grains (Sathe et al., 1992). Factors such as the type of rocks, pH and redox potential in the soil, the existence of some organic and inorganic compounds, soil moisture and salinity, soil sulphate concentration, plant species, soil-management practices, oxidation state of the element (the absorption of Se^{+6} is higher than that of Se^{+4}), nature of draining waters, and climatic conditions all influence the distribution, status and availability of this element (Combs, 2001; Aro et al., 1995; Barclay et al., 1995).

The food chain affects in the same manner the Se status in human populations (Burk and Levander, 2002; Goyer and Clarkson, 2001). The type of the soil diversifies the Se content. In acid soils Se is mainly present as selenite which has very low solubility and plant availability. In alkaline soils, Se is oxidized to selenate, which is more soluble and more available for uptake in the crops. There are some zones where Se levels in soil are very low (≤0.05 ppm), such as parts of China, Finland and New Zealand. In these regions, diseases caused by Se deficiency in livestock and the effect on human health are well known. Nevertheless, in regions of high Se soil concentrations (≥5 ppm), there is a net excess of this element as observed in Canada, Ireland, some regions of the western USA, and some zones of China, France, Germany, etc (Aro et al., 1995; Simonoff and Simonoff, 1991).

Food protein content is another important factor influencing Se presence in food since due to their physicochemical similarity Se can replace sulphur in the amino acids as Se-Met, and selenocysteine (Se-Cys) (Navarro-Alarcon and López-Martínez, 2000; Simonoff and Simonoff, 1991). Additionally, selenocompounds would be used in the synthesis of Se-amino acids (mainly, Se-Met and Se-Cys), and finally incorporated in vegetable proteins. So, the Se forms included in the vegetable proteins of animal feed would ultimately be employed in the synthesis of the animal's own proteins, facilitating their accumulation in livestock. Most plants are not capable of accumulating large amounts of Se (concentrations rarely exceed 100 μg g^{-1}, dry weight). However, various plant species such as garlic (*Allium sativum*), Indian mustard (*Brassica juncea*), canola (*Brassica napus*), and some mushrooms have been recognized as Se

accumulators. They have the ability to take up large amounts of Se (>1000 mg Se kg^{-1}) without exhibiting any negative effects (Dumont et al., 2006).

As a conclusion, diet is the major source of Se and intake of this essential element depends on its concentration in food and amount of food consumed (Navarro-Alarcon et al., 2005). Combs (2001) indicated that an adequate adult diet should have at least 40 μg day^{-1} of Se to support the maximum expression of Se enzymes and perhaps as much as 300μg day^{-1} to avoid at a large scale cancer risk. Approximately 80% of dietary Se is absorbed, although this figure depends on the types of food consumed. Overall absorption of all forms of Se is relatively high (70–95%), but varies according to the source and the Se status of the subject. Wheat and meats are the most important Se dietary sources. Selenium tends to be present in relatively high concentrations and, compared with Se salts, Se in these foods is highly bioavailable (Finley, 2006).

1.3.2. Selenium in food of animal origin

Data on Se content in different foods such as meat, chicken, fish and eggs showed that they are protein-rich foods containing high levels of Se (Sirichakwal et al., 2005; Klapec et al., 2004). In these food groups, Ventura et al. (2007) encountered Se levels ranging from 87.6 to 737 ng g^{-1}. Fish and eggs showed the highest Se concentration (Haratake et al., 2007; Pappa et al., 2006). Meat, fish and eggs contribute the major part of dietary Se in several countries such as Greece, Portugal and Japan (Haratake et al., 2007; Ventura et al., 2007; Pappa et al., 2006). In Japan, fish was the greatest Se contributor (up to 60% of daily total intake) rather than the staple foods (rice and vegetables) (Haratake et al., 2007). Tinggi (1999) reported Se content in eggs from Australia to have a mean concentration of 90ng g^{-1} in white and 260 ng g^{-1} in yolk (boiled eggs). Marzec et al. (2002) reported that Se levels in meat products ranged from 55 to 329ng g^{-1}. These values were higher than those for the other food groups. Meat showed large variations in Se concentration, reflecting the differences in Se concentrations of the feed consumed by the animals (Pappa et al., 2006; Naughton and Marks, 2002). According to Pappa et al., 2006 mean

concentrations of Se in meat from Greece ranged from 48.8 to 94.1ng g^{-1}, with pork measuring significantly higher than beef.

1.3.3. Selenium's bioavailability

The most essential characteristic of Se is that its deficiency and toxic concentrations are very close to each other. It must always be taken into consideration that its content in any given food is unreliable and of course not to forget its bioavailability. The right balance always has to be determined as to avoid deficiency or abundance. The quantity absorbed by an organism plays an important role since only a fraction of it is transformed into a biologically available form in the end (Cabañero et al., 2007; Cabrera et al., 1996). Ideally, only a complete evaluation of bioavailability involving measurements of total nutrient content, absorbable fraction, amount actually absorbed, and percentage utilized by the organism could determine all the parameters needed during experiments but *in vivo* that possibility is limited because of the cost and excessive labor necessary (Cabañero et al., 2007).

Selenium's bioavailability strongly depends on the chemical form of Se found in the food. Specifically, selenocompounds identified in plants include selenate, selenite, selenocystine, Se-Met, selenohomocysteine, Se-methylselenocysteine, γ-glutamil-selenocystathionine, Se-Met selenoxide, γ-glutamyl-Se-methylselenocysteine, selenocysteineselenic acid, Se-propionylselenocysteine selenoxide, Se-methyl selenomethionine, selenocystathionine, dimethyl diselenide, selenosinigrin, selenopeptide and selenowax. However, the presence of Se-Cys in plants is still controversial (Whanger, 2002). On the other hand, selenocompounds in animal tissues are Se-Cys, selenotrisulfides of cystine, selenate and selenite. Se bioavailability is affected by its chemical form. As already stated, generally, organic compounds of Se are more bioavailable than the inorganic forms (Thomson, 2004).

Selenium concentration in soils varies significantly (Reilly, 1996); and its availability to plants depends on many factors. In the case of acidic soil pH or poor soil aeration, Se can form insoluble complexes with iron hydroxide and become poorly available. Furthermore, the amount

of water-soluble Se (available for plants) in soils varies substantially and does not correlate with total soil Se (Combs and Combs, 1986).

Several studies have shown that Se bioavailability in meat is high because Se forms in foods of animal origin are mostly Se-Cys and Se-Met (Dumont et al., 2006). Se-Met is an essential seleno-amino acid, which is the major nutritional source of Se for animals, and it is known to be highly bioavailable. Fox, et al. (2004) indicated that Se in fish is a highly bioavailable source of dietary Se, and that cooking the fish did not affect Se absorption or retention. These authors also observed that Se from yeast is less bioavailable. Finley (2006) observed that reports on Se bioavailability from yeast are mixed; one group reported that Se from yeast was effective for increasing the concentration of Se in red blood cells, but compared with selenite and selenate, was ineffective in increasing GPx activity. In contrary, another group reported that Se from yeast was almost twice as bioavailable as Se from selenite and selenate for restoration of depleted GPx activity. These discrepancies may reflect differences in the study populations as well as a difference in the chemical speciation of Se in yeast. Finley et al. (2004) concluded that the chemical forms of Se species also differ among foods. For example, in broccoli, which is a Se-accumulating plant that contains many methylated forms of Se, its bioavailability has been reported to be low.

However, red meats such as pork or beef could accumulate Se when the animal is fed high Se diets, and Se from such meats has been reported to be highly bioavailable for selenoprotein synthesis. In general, animal trials demonstrated that bioavailability of organic Se (Se-Met and Se-yeast) was higher than inorganic forms (selenite and selenate). The same trend was observed in human studies (Dumont et al., 2006; Lacour et al., 2004).

Selenium availability from various plant-based sources also varies. For example, Se from yeast, wheat and alfalfa is highly available, while Se in most other plant sources is moderately available. Selenium in fish meat is poorly available (Cantor, 1997) despite the fact that Se content of fish meat can be quite high.

Finally, in human liver cells the highest concentrations of Se were found in the mitochondrial and nuclear fractions (Chen, 1999). In contrast, Se in bovine kidney was mainly concentrated in the nuclear and cytosolic fractions (Jayawickreme and Chatt, 1987). Se-containing proteins differed in their distribution among liver subcellular fractions (Chen, 1999)

indicating the possibility of different metabolic pathways for Se incorporation into tissue proteins.

1.3.4. Selenium metabolism

Recent advances in Se biochemistry have provided a deeper understanding of the principal differences in metabolism of the two forms of Se namely inorganic Se (sodium selenite or selenate) and organic Se (mainly selenomethionine). Organic Se, which can be found in grains, forages and other feed ingredients, is primarily in the form of selenomethionine (Se-Met) and is metabolized in the same way as methionine (Wolfram, 1999). It is actively transported through intestinal membranes during absorption and actively accumulated in such tissues as liver and muscle. Selenomethionine is not synthesized in animals or humans and must be derived from feed sources (Schrauzer, 2000). In contrast, while inorganic Se is absorbed as a mineral, little is retained in tissue reserves. A large part of inorganic Se is excreted with faeces in ruminants or with urine/urates in non-ruminants with little entering body proteins (Wolfram, 1999).

Furthermore, it is known that Se-Cys is the major selenocompound present in selenoproteins of body tissues (Sunde, 2000). The total amount of Se in the human body varies from 10 to 20 mg. Fifty percent of body Se is located in the skeletal muscles, although organs like the kidneys, testes and liver have the highest relative concentration of Se. On the other hand, cells that reveal a higher consumption of Se are those of the immune system, erythrocytes and platelets. Se is mainly eliminated from the body by urine, although significant losses via faeces also occurs (Burk and Levander, 2002). Additionally, low amounts of Se are lost through the skin and respiration.

It is known that several Se forms present in foods are usually well absorbed in the human intestine (the usual absorption rate ranges from 50 to 100%) (Navarro-Alarcon et al., 2005; Sunde, 2000). Se-Met is absorbed by the same active transport mechanism used by methionine because Se can substitute for sulphide atoms due to its similar ionic radius.

The selenate is actively absorbed by a mechanism common to sulphate, depending on the Na^+ gradient and maintained by the Na^+/K^+ ATPase. On the other hand, Se-Cys and selenite are

not absorbed by active transport and their capture is not inhibited by similar sulphur compounds or by body Se status (Mataix Verdu and Llopis, 2002). Several selenocompounds exist in animal and plant tissues (Gard, 2008). Specifically, selenate is the major inorganic selenocompound found in both animal and plant tissues (Whanger, 2002).

On the other hand, Se-Met is the predominant selenocompound in cereal grains, grassland, legumes and soybeans, and, in some cases, Se-enriched yeast. Finally, Se-methylselenocysteine is the major selenocompound in Se enriched plants such as garlic, onions, broccoli flowers and sprouts, and wild leeks (Whanger, 2002). Se-Cys, mainly from meat, is directly used in the GPx synthesis. Nevertheless, Se-Met from plants can directly replace methionine amino acid during the synthesis of Se-containing proteins. On the other hand, selenate and selenite incorporate directly into the Se pool when used in synthesis of specific selenoproteins and Se-containing proteins, independent of their origin (animal or vegetable) (Navarro-Alarcon et al., 2005; Mataix Verdu and Llopis, 2002; Brody, 1999).

As a conclusion, the human body metabolizes the various Se forms into selenide as HSe^- which seems to be the common point for regulating Se metabolism (Navarro-Alarcon et al., 2005; Burk and Levander, 2002; Mataix Verdu and Llopis, 2002; Brody, 1999). It has been found that animals synthesize many different intermediary metabolites during the conversion of inorganic Se to organic forms or vice versa (Ganther, 1999). As mentioned above, HSe^- ion is a key metabolite formed from inorganic sodium selenite via selenodiglutathione through reduction by thiols and NADPH-dependent reductases and released from Se-Cys by lyase action (Bjornstedt et al., 1992). Serum and plasma Se levels depend on the Se bioavailable fraction present in diet. In plasma, two selenoproteins have been cited as extracellular carriers of Se, namely selenoprotein P and GPx-3. However both of these selenoproteins contain Se as Se-Cys making neither of them likely carriers of Se. Nevertheless, low molecular weight forms of Se have been identified as possible Se carriers in plasma.

The specific role of the chick duodenum in the assimilation of Se (selenate or selenite) was shown by Apsite (1993). Selenite is passively absorbed in the intestine with highest concentrations found in duodenum, liver and kidneys (Apsite, 1994). Absorption of Se was found to be greatest in the duodenum and anterior ileum of the chicken (Pesti and Combs, 1976). Absorption of ^{75}Se from selenite, selenate, and Se-Met was determined in ligated loops from duodena, jejuna, and ilea of Se-deficient rats (0.009 ppm Se) or rats fed selenite-supplemented

diets (0.20 ppm Se) (Vendel, 1992). Selenium deficiency had no effect on absorption of any selenocompound in any intestinal segment. Selenomethionine was absorbed from all segments. In contrast, selenate and selenite were most efficiently absorbed from the ileum. In studies investigating the relationship between vitamin E/Se and glucose absorption, Se plus vitamin E or Se alone modified jejunal glucose absorption, depending on the duration of feeding and age of chickens. In younger chickens, providing the supplements for 11 days depressed glucose absorption, but when the supplements were given for 18 days, glucose absorption was increased. In older chickens, Se alone or with vitamin E given for 13 days increased glucose absorption (Giurgea and Roman, 1992).

Absorption studies with ligated duodenal loops or oral doses indicated that high vitamin A intake (Combs, 1976) or dietary ascorbic acid (Combs and Pesti, 1976) promoted the enteric absorption of selenium. Ethoxyquin (an antioxidant used as a food preservative and a pesticide) was effective in alleviating exudative diathesis (a disease caused by a nutritional deficiency of vitamin E, characterized by severe edema of the subcutaneous tissues) when fed separately from Se. It was also effective in promoting increases in plasma GSH-Px (Combs, 1978). However, transport of ^{75}Se was inhibited when ascorbic acid and selenite were injected directly into ligated duodenal loops of anaesthetized chickens. The mechanism of the inhibition seemed to be precipitation of Se within the intestinal lumen, since less ^{75}Se was found in the supernatant fraction of the luminal fluid when ascorbic acid was present (Mykkänen, 1983).

It could be suggested that evolution was the reason according to which animals, including birds, are able to metabolize Se-Met. In this respect antioxidant properties of Se-Met per se could be of great value during feed digestion. On the other hand, inorganic Se is not a part of the natural diet and in some conditions sodium selenite could be pro-oxidative. The greatest discrepancy occurs between sources containing Se as selenite compared with Se-Met, due to a difference in the manner in which they are metabolized to selenocysteine for incorporation into GSH-Px. In addition, Se-Met can be incorporated directly into body proteins and stored as such, thereby inflating bioavailability estimates based on body retention (Henry, 1995).

1.3.5 Protective role of selenium

Selenium was not even discovered as an essential nutrient until 1979 and only in 1990 was an RDA (Recommended Dietary Allowance) proposed. Its key role in the antioxidant defense system has been confirmed as it is closely associated with the population's health. Due to its overlapping function with vitamin E its importance for mammals was unknown in the earlier years (Strain and Cashman, 2002). Its protective role expands in a wide range of body functions. 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). Selenium is a component of several selenoproteins with essential biological functions (Cauwenbergh et al., 2004). This element acts as a cofactor of the GPx family of enzymes which protect against oxidative stress. Specifically, Se-dependent GPx enzyme recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides, including hydrogen peroxide. In general, all these enzymes at their reduced state catalyze the breakdown of lipid hydroperoxides and hydrogen peroxides in human cells (Hartikainen, 2005; Navarro-Alarcon, 2005; Cauwenbergh et al., 2004; Navarro-Alarcon et al., 2000). Moreover, the antioxidative function of Se can help to ameliorate the damage induced by the ultraviolet-β radiation in humans.

Moreover, the selenoprotein P is a plasma protein highly expressed in the liver and kidney. This protein constitutes the main plasma Se carrier carrying more than 60% of plasma Se. Besides, it is known that the protein levels depend on the body's Se status, such that it has been used as a biomarker of body Se content. Particularly, the selenoprotein P acts as an extra cellular antioxidant associated with the vascular endothelium which diminishes the peroxinitrile ($ONOO^-$) level that represents reactive nitrogen specie (Li et al., 2007). Several studies have also found that Se protects animals against toxicity associated with high exposure and/or intake of heavy metals like mercury, lead, cadmium and silver (Cabañero et al., 2007; Kibriya et al., 2007; Mousa et al., 2007; Navarro-Alarcon et al., 2005; Thorne, 2003).

Biological availability of Se for prevention of exudative diathesis was taken as 100% for sodium selenite. It ranged between another forms of Se from 74% for sodium selenate to 7% for elemental Se, in plant feedstuffs from 210% for lucerne meal to 60% for soybean meal and in animal feedstuffs from 25% for herring meal to 8.5% for fish solubles (Cantor, 1975; Cantor and

Scott, 1974). However, Se-Met was four times as effective as either selenite or selenocystine with respect to prevention of pancreatic degeneration and increasing the relative weight and Se concentration of the pancreas (Cantor, 1975).

In farm animals, diseases associated with Se deficiency have been an important problem. White muscle disease is a nutritional muscular dystrophy that is the most common concerning Se deficiency disease (Peter and Costa, 1992). Deprivation of Se is associated with reduced antioxidant protection, redox regulation and energy production as a consequence of suboptimal expression of one or more of the Se-containing enzymes (Thomson, 2004). At the same time, supranutritional intakes of Se (more than required for Se-Cys enzyme expression) appear to reduce cancer risk (Combs, 2001). Hamilton (2004) reported the existence of three Se levels of biological activity: (1) trace concentrations are required for normal growth and development; (2) moderate concentrations can be stored and homeostatic functions maintained; and (3) elevated concentrations can result in toxic effects. Accordingly, low Se status is likely to contribute to morbidity and mortality due to infectious as well as chronic diseases, and increasing Se intakes in all parts of the world can be expected to reduce cancer rates (Tinggi, 2003).

1.3.6. Selenium supplementation

Concerning selenium's protective role for the organism, several authors considered that Se supplementation can be beneficial for individuals in regions with very low environmental Se levels (Grashorn, 2006). The major Se supplements in use for the last 20 years are selenite and selenate - both inorganic forms of Se. The limitations of using inorganic Se are also well known: toxicity, interactions with other minerals, low efficiency of transfer to milk, meat and eggs and an inability to supply and maintain Se reserves in the body. As a result, a high proportion of the element consumed in inorganic form is simply excreted. Further, a pro-oxidant effect of the selenite ion (Spallholz, 1997) is a great disadvantage as well. In contrast, Se-Met itself is considered to possess antioxidant properties (Schrauzer, 2000). While recent developments suggest that sodium selenite was ultimately not the best choice for Se supplementation, when Se addition to animal diets first began, it was both economic and greatly helped to solve problems

associated with Se deficiency. Thus, the use of sodium selenite in animal diets has recently been questioned (Pehrson, 1993).

In some areas where soil Se is low, different strategies have been followed to supply the population with sufficient Se: (1) Use of Se-enriched fertilizers: In order to reach Se RDAs, some countries like Finland, for example, decided in 1984 to add sodium selenate to farmlands (Varo et al., 1988). Hartikainen (2005) indicated that this supplement positively affects not only the nutritive value of the entire food chain (soil to plants to animals to humans) but also improves plant yields. (2) Supplementation of farm animals with Se. The need for Se has resulted in the rise of direct Se enrichment of certain foods, such as using Se-enriched fertilizer. However, part of the Se in these food products is lost (volatilization, degradation) during harvesting and manipulation prior to consumption (Dumont et al., 2006).

In Australia, subclinical Se deficiency has largely been eliminated as a result of intervention programs which give Se supplements to animals. Tinggi (2003) reported a number of Australian Se supplement strategies to increase Se in farm animals. These strategies include: a) direct application of Se to pastures to increase Se uptake by plants for animal feed; b) supply of sodium selenite or selenate incorporated into salt blocks or licks; c) direct administration of Se to animals by drenching with Se salt solutions such as sodium selenite; and d) the use of Se pellets that slowly release Se in the animal's intestine.

Bourre and Galea (2006) described a new natural multi-enriched egg as an important source of omega-3 fatty acids, vitamins D and E, carotenoids, iodine, and selenium (45% RDA). These authors remarked that these eggs are beneficial for everyone and particularly appropriate for older people. Additionally, in Greece, a new brand of Se-enriched eggs called Vi-Omega-3 was developed, delivering 22 μg Se with a single egg (Pappa et al., 2006).

Muñiz-Naveiro et al. (2006) indicated that it is possible to obtain Se-enriched cow milk at different concentrations without altering the original composition of the milk. Lyons et al. (2007) remarked that optimizing Se nutrition for poultry and farm animals increased efficiency of egg, meat and milk production and, more importantly, improved quality. Considering all the above, as well as the recent advances in genomics and proteomics, along with the newly described selenoproteins, a driving force in reconsidering old approaches to Se nutrition is foreseen (Kellof et al., 2000).

1.3.7. Assessment of body nutritional status of selenium

When a Se deficiency is established, the activity of Se dependent enzymes diminishes depending on the enzyme type and body tissue. Of all the enzymes, the activities of the plasmatic and hepatic GPxs are the most dependent on the Se supply. The nutritional Se status can be assessed by several human fluids and tissues (whole blood, plasma, serum, hair and toenails). In fact, in most studies the Se status has been assessed by measuring the element either in serum or in plasma erythrocytes, platelets or whole blood, and by determining the GPx activity in whole blood or platelets. Recently, levels of selenoprotein P, a Se-rich protein mainly present in plasma, have also indicated successfully the Se status in human beings (Persson-Moschos et al., 1995).

Human nail clippings have also been employed in epidemiological studies as indicators of Se exposure (Slotnick and Nriagu, 2006). It is believed that nail clippings show the exposure that occurred over the past 6 to 12 months. On the contrary, blood and urine are markers of shorter exposure periods (Slotnick and Nriagu, 2006; Navarro-Alarcon and López-Martínez, 2000) which means that urine and blood Se levels show recent intake for no longer than several days for urine or several weeks for blood-based measurements, respectively. Se urinary excretion is closely correlated with plasma and serum and could be used to monitor recent dietary intake of Se. Thomson (1998) reported that Se urinary excretion constitutes between 50 and 60% of the total amount excreted, so dietary intake could be estimated simply by multiplying by two the daily urinary excretion of Se.

1.4. Selenium – Cadmium interaction

In the past few years, increasing consideration has been given to interactions occurring in the organism between toxic metals and elements essential for life. These interactions are

complex and involve biometals such as zinc (Zn), copper (Cu), iron (Fe), selenium (Se), calcium (Ca) and toxic elements, including cadmium (Cd) (Ninomiya et al., 1993; Tandon et al., 1994).

Selenium is generally considered protective against cadmium toxicity (Jamba et al., 1977) and because of its high affinity for cadmium, the formation of a Cd-Se protein complex in the prostate could account for its protective effect against prostate cancer development. That supplemental selenium significantly reduces prostate cancer risk was one of the key results of the Nutritional Cancer prevention (NCP) Trial (Clark et al., 1996).

1.4.1. Interactions between Se, Cd and other trace elements

Chemical interactions between toxic and essential trace elements have been investigated in animals exposed to relatively high levels of contaminants (Spierenburg et al., 1988; Koh and Judson, 1986) or in experimental studies (Groten et al., 1991; Reddy et al., 1987). The absorption, accumulation and toxicity of each element may be affected by diverse factors, including interactions with other elements. Indeed, interactions between toxic and essential metals are vital to mineral balance and the antioxidant defense system (Pappas et al., 2010a, 2010b; Lopez-Alonso et al., 2007). However, in spite of it, it is well documented that interactions between metals can occur at levels of exposure well below those at which toxicity may be detected (López Alonso et al., 2002a; Goyer ,1997). There is little information on the interactions between metals at the low level of metal exposure that usually occurs in nature. When it exits, it is generally limited to a few numbers of trace elements, mainly copper, zinc and iron.

We cannot fully understand the molecular mechanisms of most of these metal interactions which cause the necessity to elucidate these chemical interactions (Rahil-Khazen et al., 2002). Though increasing evidence supports the important role of metals in neurobiology and many diseases (Patriarca et al., 1998; WHO, 1996). The accumulation of any of these elements (As, Cd, Cu, Pb, Se and Zn) in internal organs of cattle and other animals can often be mediated by the absorption and metabolic cycle of other elements (Coudray et al., 2006; Custer et al.,

2004; Dorton et al., 2003; Frank et al., 2000; Bebe and Panemangalore, 1996; Abdelrahman and Kincaid, 1992).

Liver and kidney are the only organs able to metabolize trace elements. Nevertheless, it is recognized that they are also extremely vulnerable to metal accumulation and toxicity, which is why these interactions must be studied through these tissues (Rahil-Khazen et al., 2002). Unfortunately, non-lethal samples like blood or urine can only provide us with a rough indication of the total amount of a trace metal in the body but give no information about the distribution and interaction of the metals in different tissues.

1.4.2. Selenium and zinc against cadmium

It is well known that many of the toxic effects of Cd result from interactions with essential elements such as zinc (Zn) and selenium (Se). These interactions can take place at different stages of the absorption, distribution, and excretion of trace elements, and they can affect the biological functions of such elements as well (Brzóska et al., 2001). Se is a vital trace element which, in mammals, exerts its most important function via selenium-dependant glutathione peroxidase (GSH-Px). Se deficiency is usually associated with increased lipid peroxidation which alters the integrity of cell membranes and, consequently, affects cell functions (Valko et al., 2005). Zn acts as an antioxidant, since it is involved in cell membrane stabilization, copper/zinc superoxide dismutase (Cu/Zn SOD) structure, and metallothionein induction. Zn deficiency can cause compromised antioxidant defense system, which in turn can be reflected by evidence of cellular or tissue oxidative damage (Oteiza et al., 1999). The treatment with Zn (Chowdhury et al., 1987; Jemai et al., 2007) or Se (Jamba et al., 2000; Newairy et al., 2007) during Cd exposure has been demonstrated to have protective effects on Cd-induced toxicity in various organs and tissues.

1.4.3. Interactions with cadmium

Interactions have been reported between cadmium and zinc (Spierenburg et al., 1988; Nicholson et al., 1984). Firstly, incorporation of cadmium in the sulfhydryl (cysteine)-rich protein MT is one of the principal detoxification mechanisms against the metal. MT is specific not only for binding cadmium but also for binding copper, mercury, silver, and zinc. In fact, MT synthesis is induced by the presence of these metals as evidenced by increases in MT mRNA (Bremner and Davies, 1975). The main physiologic role of MT is to act as a homeostatic control mechanism by controlling the metabolism of both copper and zinc.

Therefore, binding of a nonessential metal such as cadmium to MT is a fortuitous result of the chemical similarity between cadmium and the essential trace metals. Cadmium has a higher affinity for MT and in fact displaces zinc from the cysteine binding sites but does not displace copper because this essential trace metal has higher MT affinity (Funk et al., 1987). The free essential trace metals could then stimulate the production of more MT or be excreted in the urine, which would explain the deficiencies in these two metals observed during cadmium exposure. Zinc and copper supplements are thought to act by inducing MT synthesis, which allows the body to be adequately supplied with enough MT to combat the cadmium insult. Even though MT offers mostly protective effects against cadmium toxicity, there is evidence that it is indirectly involved in contributing to cadmium's main toxic effect: renal failure. In the urine of cadmium-dosed animals, a large amount of cadmium-MT complex was recovered, indicating that the kidney is not very efficient at MT reabsorption (Cherian et al., 1975).

This complex is somewhat retained by the kidney and the protein is rapidly degraded, leaving free cadmium to accumulate in the kidneys. Consequently, MT is the vehicle that transports cadmium to its toxic site of action, the kidney. Additionally, it has been demonstrated that toxic metals can disrupt trace element metabolism. Cadmium toxicity affects Ca metabolism either by direct toxicity to bone or indirectly from renal toxicity (Pappas et al., 2010a; Goyer, 1997; Webb 1979).

Cadmium and Pb also have many similarities in kinetic, metabolic, and toxic manifestations. The two metals bind with similar bio-active ligands (-SH, $-CH_2$ and $-NH_2$) and compete with the same essential elements such as cadmium, iron, zinc, etc. (Klaasen, 1991).

They are stored in the liver, kidney, and bone sites, and eliminated by renal clearance (Haneef et al., 1998). The effect of lead is actually similar to cadmium (Rudy, 2009). Lead is a well-documented metal toxicant, exposure to which leads to many fatal diseases, including the dysfunction of renal-, blood- and neurological systems (Mittal and Goyal, 1995).

1.4.4. Interactions with selenium

Selenium interacts with several trace elements, and these interactions can be additive, antagonistic, or synergistic, and in some cases they reverse the interaction, i.e. antagonism changed to synergism (Akl et al., 2006; Hamilton, 2004). 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).

Perhaps one of the most commonly reported interactions between inorganic elements is the antagonistic interaction between Hg and Se. Se is recognized to decrease Hg toxicity when both elements are simultaneously administrated (Cabañero et al., 2007; Caurant et al., 1996). Another metal interaction that has been described in the literature is the one between selenium and copper. Experimental studies have demonstrated that high copper levels in one's diet induce important oxidative changes, and these changes can be modulated with added selenium (Gawthorne, 1987).

1.4.5. Interactions between trace elements

There is a strong relationship between cadmium and zinc in the kidney and in the liver of the general population probably because of a link in their metabolism in these tissues that involves metallothionein which acts as a common ligand for both metals (Terra et al., 1994). Similarly to Cu and Zn, iron's ability to accept or donate electrons makes it essential to the catalytic activity of many redox-active enzymes. There have been many reports of interactions between copper and other elements in cattle and other livestock species. An early finding was the interaction between copper and iron, related to hemoglobin synthesis (Hart et al., 1928). Three-way interactions between copper, molybdenum and sulphur have also been reported (Underwood and Suttle, 1999), so that the daily copper requirements of cattle are strongly dependent on molybdenum and sulphur levels in the diet.

There are many examples of lead-copper interactions in the scientific literature shown through various experiments. In experimental studies in mice, administration of a high-lead diet has been reported to lead to a deficiency in copper absorption (Dhawan et al., 1995), and conversely adding copper to the diet has been found to increase lead accumulation (Cerkleroski and Forbes, 1977). Conflicting results have been obtained in cattle studies. Some studies have described a negative association between these elements in animals with low lead levels (Miranda et al., 2005), while other studies have found no statistically significant association (López Alonso et al., 2002), or have observed a strong positive association in cattle with normal levels of copper exposure (López Alonso et al., 2004). A post-intake interaction between these two elements cannot be positively affirmed yet.

Concerning the essential metals, there have been numerous previous reports of negative associations between copper levels and levels of molybdenum (Telfer et al., 2004; Underwood and Suttle, 1999), and it has been demonstrated that levels of molybdenum determine copper requirements. It is well established that molybdenum and sulphur form complexes within the rumen that show high affinity for copper, reducing the copper available for its intestinal absorption (Telfer et al., 2004; Suttle, 1991). Previous studies have clearly demonstrated hypocupraemia and reduced hepatic copper levels in cattle supplemented with molybdenum and sulphur (Humphries et al., 1983). Mackenzie et al. (1997) reported that the copper concentrations

were positively associated with molybdenum in the liver and kidney which indicates that molybdenum does not have a significant antagonistic effect on intestinal absorption of copper in these animals since, if this was the case, these associations would be negative.

1.5. Zinc

Zinc (atomic number 30; relative atomic mass 65.38) is a metallic element belonging to group IIb and the fourth period of the periodic table. Zinc is a chalcophilic element like copper and lead, and a trace constituent in most rocks. Zinc rarely occurs naturally in its metallic state, but many minerals contain zinc as a major component from which the metal may be economically recovered. Sphalerite (ZnS) is the most important ore mineral and the principal source for zinc production (WHO, 2001a). For non-contaminated soils worldwide, Adriano (1986) reported average zinc concentrations of 40–90 mg kg^{-1}, with a minimum of 1 mg kg^{-1} and a maximum of 2000 mg kg^{-1}. Low levels are found in sandy soils (10–30 mg kg^{-1}), while high contents are found in clays (95 mg kg^{-1}) (Adriano, 1986).

Zinc is fundamental for cell growth, development and differentiation, but the biochemical mechanisms involved are not totally known. Zinc interacts with general metabolism of protein, carbohydrate and lipid, as well as on taste, smell, appetite regulation and food consumption. On the other hand, it is closely involved in the synthesis and action of growth hormone (GH), somatomedin-C, alkaline phosphatase, collagen, and osteocalcin. In addition to GH, there are interrelationships between zinc and other hormones such as testosterone, thyroid hormones, insulin, and vitamin D. This micronutrient participates both in the synthesis and actions of these hormones, which are intimately linked to bone metabolism. Thus, in all these events zinc acts positively on growth and development.

Furthermore, zinc is necessary for the function of a large number of metalloenzymes, including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase, leucine aminopeptidase, and superoxide dismutase. Zinc deficiency has been associated with dermatitis, anorexia, growth retardation, poor wound healing, hypogonadism with impaired reproductive capacity, impaired immune function, and depressed mental function. An increased incidence of

congenital malformations in infants has also been associated with zinc deficiency in the mothers. Zinc deficiency may also have an impact on the carcinogenesis of other chemicals, although the direction of the influence seems to vary with the carcinogenic agent (Institute of Medicine, 2001).

The effects of inhalation exposure to zinc and zinc compounds vary somewhat with the chemical form of the zinc compound, but the majority of the effects seen will occur within the respiratory tract. Following inhalation of zinc oxide, and to a lesser extent zinc metal and many other zinc compounds, the most commonly reported effect is the development of "metal fume fever." Metal fume fever, a well-documented acute disease induced by inhalation of metal oxides, especially zinc, impairs pulmonary function but does not usually progress to chronic lung disease. Symptoms generally appear within a few hours after acute exposure, usually with dryness of the throat and coughing. The most prominent respiratory effects of metal fume fever are substernal chest pain, cough, and dyspnoe. The impairment of pulmonary function is characterized by reduced lung volumes and a decreased diffusing capacity of carbon monoxide. Leukocytosis persisting for approximately 12 hours after the fever dissipates is also a common manifestation of metal fume fever. In general, the symptoms of metal fume fever resolve within 1–4 days after cessation of exposure and do not lead to long-term respiratory effects. The exact mechanism behind the development of metal fume fever is not known, but it is believed to involve an immune response to the inhaled zinc oxide (Institute of Medicine, 2001).

It has been suggested that the zinc oxide causes inflammation of the respiratory tract and the release of histamine or histamine-like substances. In response, an allergen-antibody complex is formed that may elicit an allergic reaction upon subsequent exposure to the allergen. In response to the allergen-antibody complex, an anti-antibody is formed. The anti-antibody dominates with continued exposure to the zinc oxide, thereby producing a tolerance. When the exposure is interrupted and re-exposure occurs, the allergen-antibody complex dominates, producing an allergic reaction and symptoms of metal fume fever (Malo et al., 1990; Lindahl et al., 1998). Nausea has been reported by humans exposed to high concentrations of zinc oxide fumes (300–600 mg m^{-3}) and zinc chloride (~120 mg m^{-3}) smoke, as well as following oral exposure to zinc chloride and zinc sulfate.

Other gastrointestinal symptoms reported in cases of excessive zinc exposure include vomiting, abdominal cramps, and diarrhea, in several cases with blood. In general, oral exposure

levels associated with gastrointestinal effects of zinc have not been reliably reported, but the limited available data suggest that oral concentrations of 910 mg l^{-1} or single-dose exposures of ~140–560 mg (acute oral doses of 2–8 mg kg^{-1} day^{-1}) are sufficient to cause these effects. The noted effects are consistent with gastrointestinal irritation. It is unclear in the majority of human studies whether the gastrointestinal effects seen following zinc inhalation were due to systemic zinc or was the result of direct contact with the gastrointestinal tract following mucociliary clearance of inhaled zinc particles and subsequent swallowing (WHO, 2001a; ATSDR, 2005). Following longer-term exposure to lower doses (~0.5–2 mg kg^{-1} day^{-1}) of zinc compounds, the observed symptoms generally result from a decreased absorption of copper from the diet, leading to early symptoms of copper deficiency.

When ingested zinc levels are very high, zinc is believed to inhibit copper absorption through interaction with metallothionein at the brush border of the intestinal lumen. Both copper and zinc appear to bind to the same metallothionein protein; however, copper has a higher affinity for metallothionein than zinc and displaces zinc from metallothionein protein. Copper complexed with metallothionein is retained in the mucosal cell, relatively unavailable for transfer to plasma, and is excreted in the faeces when the mucosal cells are sloughed off. Thus, an excess of zinc can result in a decreased availability of dietary copper, and the development of copper deficiency. This fact has been used therapeutically in the treatment of Wilson's disease. Zinc supplementation is used to substantially decrease the absorption of copper through the diet, which can aggravate the disease. Copper is incorporated into metalloenzymes involved in hemoglobin formation, carbohydrate metabolism, catecholamine biosynthesis, and cross-linking of collagen, elastin, and hair keratin.

The copper-dependent enzymes, which include cytochrome C oxidase, superoxide dismutase, ferroxidases, monoamine oxidase, and dopamine β-monooxygenase, function mainly to reduce molecular oxygen. Excess zinc may alter the levels or activity of these enzymes before the more severe symptoms of copper deficiency, which include anemia and leucopenia, begin to manifest. Numerous studies in humans receiving 40–50 mg zinc day^{-1} (0.68–0.83 mg kg^{-1} day^{-1}) have reported decreases in erythrocyte superoxide dismutase, mononuclear white cell 5'-nucleotidase, and plasma 5'-nucleotidase activities (Hewitt, 1988; ATSDR, 2005).

High-dose zinc administration has also resulted in reductions in leukocyte number and function. Some studies have also found decreases in high-density lipoprotein (HDL) levels in

humans exposed to increased levels of zinc; however, not all studies have confirmed this observation. Long-term consumption of excess zinc may also result in decreased iron stores, although the mechanism behind this effect is not presently clear. In most cases, dermal exposure to zinc or zinc compounds does not result in any noticeable toxic effects (Hughes and Samman, 2006).

Available studies have not presented evidence of reproductive or developmental effects in humans following inhalation of zinc compounds. Effects on reproductive or developmental end points have been noted in oral-exposure animal studies, but generally only at very high doses (>200 mg kg^{-1} day^{-1}). Available studies of zinc inducing carcinogenic effects in humans following both oral and inhalation exposure have not adequately demonstrated an increase in cancer incidence. The EPA currently classifies zinc and compounds as carcinogenicity group D (not classifiable as to human carcinogenicity) (WHO, 2001a; ATSDR, 2005).

1.5.1. Basic mechanisms of interactions between cadmium and zinc

Hill and Matrone (1970) first suggested that biologically important interactions can occur among bioelements and toxic metals with similar physical and chemical properties. There are many similarities between Cd and Zn. Cd is commonly found in Zn ores, which are the principal commercial sources of Cd. Both metals are classified, commonly with mercury (Hg), in group II B of post-transition elements of the periodic table. Cd^{2+} ions are intermediate in size between Zn^{2+} and Hg^{2+} (Jacobson and Turner, 1980). Cd^{2+} and Zn^{2+} ions have a similar electron configuration on their outer shells. Both metals have filled inner electron shells (d shells) with the outermost shell having two s electrons. The 10 available 3d orbitals are filled in the case of Zn^{2+}, whereas Cd^{2+} has filled 4d electronic states. Both elements have valencies of II as their stable form. Cd I compounds have also been isolated, but they are short living and unstable (Waalkes et al., 1992). Zn, unlike Cd, is relatively stable in its divalent state and does not undergo redox changes. Cd is generally classified as a ‘soft’ metal that is more likely to form covalent linkages with electron-donating ligands, whereas Zn is classified as ‘intermediate in softness’ (Jacobson and Turner, 1980). In biological systems Cd and Zn are linked to

macromolecules, primarily through sulphur (S), oxygen (O) and nitrogen (N) and interact readily with S-, O- and N-donors. They bind preferentially to the same proteins-albumin in the bloodstream and metallothionein (MT) and other proteins in tissues. Although both metals have a high affinity to biological structures (proteins, enzymes) containing -SH (sulphydryl) groups, the affinity of Cd to S-ligands as well as to N-donors is greater than that of Zn (Jacobson and Turner, 1980; Jones and Cherian, 1990). Thus Cd^{2+} and Zn^{2+} ions can compete for uptake into various cells and binding to intracellular sites and Cd may displace Zn in a number of biological processes (Endo et al., 1996, 1997). In this way, one of the metals can influence the uptake and action of the other, depending on their levels. The mechanisms of the interactions are widely debated, being competitive (Gachot and Poujeol, 1992; Endo et al., 1996, 1997) or non-competitive (Gachot and Poujeol, 1992), depending on the experimental model. Several studies have suggested that interactions between Cd and Zn in the organism result in a high degree from an affinity of both metals to MT and their ability to induce its synthesis. They can induce MT synthesis in various tissues, especially in the intestine, liver and kidney (Sharma et al., 1991; Liu et al., 1992, 1994; Prasad and Nath, 1995). Cd is about eight times more potent than Zn in increasing hepatic MT concentration (Eaton et al., 1980).

Studies have shown that the uptake of zinc by terrestrial plants is significantly increased at a low soil pH, but reduced when there is a high content of organic matter. Normal levels of zinc in most crops and pastures range from 10 mg kg^{-1} to 100 mg kg^{-1}. Some plant species are zinc accumulators, but the extent of the accumulation in plant tissues varies with soil properties, plant organ and tissue age. Zinc toxicity in plants generally causes disturbances in metabolism, which are different from those occurring in zinc deficiency. The critical leaf tissue concentration of zinc for an effect on growth in most species is in the range 200–300 mg kg^{-1} (WHO, 2001a).

Zinc deficiency in the diet may be more detrimental to human health. The human health effects associated with zinc deficiency are numerous, and include neurosensory changes, oligospermia, impaired neuropsychological functions, growth retardation, delayed wound healing, immune disorders and dermatitis. These conditions are generally reversible when corrected by zinc supplementation. A disproportionate intake of zinc in relation to copper has been shown to induce copper deficiency in humans, resulting in increased copper requirements, increased copper excretion and impaired copper status. Pharmacological intakes of zinc have been associated with effects ranging from leukopenia and/or hypochromic microcytic anemia to

decreases in serum HDL concentrations. These conditions were reversible upon discontinuation of zinc therapy together with copper supplementation (Institute of Medicine, 2001).

The RDA for zinc is 11 mg day^{-1} in men and 8 mg day^{-1} in women; these correspond to approximately 0.16 mg kg^{-1} day^{-1} for men and 0.13 mg kg^{-1} day^{-1} for women. Higher RDAs are recommended for women during pregnancy and lactation. The RfD is determined using the LOAEL (60 mg day^{-1}) and an uncertainty factor of 3. US EPA has established RfD for Zn of 0.3 mg kg^{-1} day^{-1} calculating with average bodyweight (70 kg) (US EPA, 2006).

1.5.2. Zinc bioavailability

It was in 1934 that zinc was shown conclusively to be required for normal growth and health in rats (Todd et al., 1934). The element is considered to be essential for plants, animals, and humans (Hambidge et al., 1986). As stated above, it activates several enzymes and is a component of many important metalloenzymes. The element is critically involved in cell replication and in the development of cartilage and bone. Signs of zinc deficiency in animals and humans include retarded growth, abnormal skeletal formation, delayed sexual development, alopecia, dermatitis, abnormal feathering, and impaired reproduction in both males and females. Fetal abnormalities occur and hatchability of eggs is reduced. Many animal diets require supplementation with zinc because of either low dietary levels or the presence of dietary factors which decrease bioavailability of the element. The critical importance of added dietary zinc for domestic animals was shown in 1955 when it was demonstrated that parakeratosis, a condition being observed in swine, was caused by inadequate dietary zinc (Tucker and Salmon, 1955). It was the practice to feed high calcium levels along with plant proteins containing phytate and this apparently reduced the bioavailability of dietary zinc to the point that a severe deficiency of the element occurred. It was soon demonstrated (O'Dell and Savage, 1957; O'Dell et al., 1958) that zinc was required for normal growth and development in poultry. Results obtained with swine and poultry probably led to the observations that zinc deficiency can occur in ruminants under grazing conditions in some areas of the world (Hambidge et al., 1986). In regard to the human population, severe zinc deficiency in men resulting in dwarfism and delayed sexual development

has been observed in certain countries in the Middle East, and it is suggested that marginal intakes of zinc may occur in certain segments of the U.S. population (NRC, 1989). The typical mixed diet of North American adults, in which approximately 70% of the zinc comes from meat or other animal products, is considered to provide adequate levels of the element to maintain normal zinc status in healthy young adults.

1.5.3. Zinc for growth

The first attempt to evaluate zinc bioavailability quantitatively used growing chicks and rats fed purified diets supplemented with graded levels of $ZnCO_3$ as the reference (O'Dell et al., 1972). Feedstuffs, including corn, soybean meal, sesame meal, and fish meal, were substituted in the basal diet to supply, by analysis, 5 to 10 ppm zinc. The data were treated as a standard curve assay. Weight gain of animals fed reference zinc was regressed against the logarithm of dietary zinc to establish the slope of the standard curve. Slope ratio assays have also been applied to growth response assays with rats (Forbes and Parker, 1977; Franz et al., 1980a,b) and chicks (Hempe, 1987). Slope ratio assays based on growth rate of rats predict higher bioavailability than those based on bone zinc (Franz et al., 1980a,b). Best fit curves can be calculated and used for estimation of bioavailability (Franz et al., 1980a,b). Some investigators regress rate of gain on total zinc consumed rather than on dietary concentration (Lo et al., 1981; Forbes et al., 1983) although this generally has little effect on the value derived.

1.5.4. Zn interactions

It is well known that zinc decreases cadmium-induced toxicity, including lethality (Gunn et al., 1968; Probst et al., 1977), testicular damage (Parizek et al., 1957; Gunn et al., 1964), carcinogenesis (Gunn et al., 1964) and cytotoxicity in hepatocytes (Stacey et al., 1981). The mechanism for the zinc tolerance to cadmium toxicity is postulated to be mediated by metallothionein (Leber et al., 1976) which sequestrates cadmium and results in less distribution

of the metal to the intracellular particulate fraction (Goering et al., 1984). However, it was reported that zinc protection may be mainly due to a decrease in cadmium accumulation in a cultured bone (Kaji et al., 1988).

Zinc is an important antioxidant (Brzoska et al., 2000; Lansdown et al., 2000).which interacts with other metals like Cd. These interactions can take place at different stages of the absorption, distribution and excretion of trace elements, as well as affecting the biological functions of such elements (Brzoska et al., 2000). Zinc is responsible for decreasing ROS production (Fernandez et al., 2003). Some studies have reported the ability of Zn to interact with essential elements such as Cu and Fe, decreasing their content in tissues and retarding the oxidative processes (Santon et al., 2003). It is involved in cell membrane stabilization, MT synthesis (Scheuhammer et al., 1985; Tandon et al., 2001) and superoxide dismutase (Cu/Zn SOD) structure. Numerous studies have shown that Zn supply may reduce Cd absorption and accumulation, and also prevent or reduce the adverse actions of Cd (Brzoska et al., 2000; Ueda et al., 1987; Dorian et al., 1995), whereas Zn deficiency can intensify Cd accumulation and toxicity (Sarkar et al., 1995; Sendelbach et al., 1989; Tang et al., 1998).

On the other hand, it was also reported that Zn provided protection against the toxicity of heavy metals such as Cd and Hg (Webb et al., 1972; Gunn et al., 1968). This Zn induced tolerance was thought to be related to MT synthesis. One possible mechanism for the protective effect is that Cd replaces Zn in Zn-methionine synthesized after Zn pretreatment (Tanaka et al., 1977).

1.5.5. Tissue concentration

Although the zinc concentration in most soft tissues varies little with zinc status, plasma and bone zinc are low in animals deprived of zinc. This has been observed in chicks (Savage et al., 1964), Japanese quail (Fox and Harrison, 1964), calves (Miller et al., 1968). Momcilovic et al. (1975) first used total femur zinc for quantitative evaluation of zinc bioavailability in rats. They introduced the slope ratio method to zinc bioavailability by regressing log of femur zinc against the dietary zinc concentration. The log function provided a linear response over a wider

range than that occurring with weight gain. This is possibly due to the fact that bones store zinc to a greater extent than soft tissues. Storage may also account for the lower bioavailability value obtained with the bone zinc assay, as observed by several investigators (Forbes and Parker, 1977; Franz et al., 1980a; Shah and Belonge, 1984). Rat bone seems to serve as a zinc sink and stores surges of readily absorbed zinc.

The plasma zinc flux probably increases markedly after a meal of readily available zinc, and a high proportion finds its way into bones since only a limited proportion can be used for growth. Thus, when bone zinc serves as the response criterion, less readily available zinc sources have lower bioavailabilities than is the case when growth rate is the criterion. For dietary concentrations of zinc that slightly exceed the growth requirement, bone zinc content is an acceptable index of bioavailability. Slope ratios have also been used in chick assays of zinc bioavailability. Hempe (1987) found a linear arithmetic response in tibia zinc as dietary zinc increased over a range of 6 to 18 ppm, and the slope ratio of soy flour to $ZnCO_3$ agreed with that obtained when weight gain was the criterion. Henry et al. (1987) analyzed tissues from chicks fed for 1 week a practical-type diet supplemented with graded levels of zinc, ranging from 500 to 1000 ppm. A linear increase in tissue concentration was observed, with bones showing the greatest sensitivity to high levels of dietary zinc.

Hahn and Baker (1993) fed grades doses of various zinc salts to young pigs consuming a basal corn-soybean diet containing 125 ppm zinc. Plasma zinc concentrations were erratic when dietary concentrations were below 1000 ppm added zinc and the reference source was $ZnSO_4.H_20$. Between 1000 and 5000 ppm however, excretion routes apparently became saturated so that plasma zinc concentration increased fourfold in a dose-dependent manner. The increase in plasma zinc was twice as great for the sulfate as for zinc oxide, and zinc-methionine produced a response greater than that of the sulfate. Feeding zinc-lysine resulted in plasma zinc concentrations similar to those in pigs fed the sulfate. These results were similar to those reported by Wedekind and Baker (1990) and Wedekind et al. (1992) in which chicks were fed supplemental zinc at low dietary concentrations and weight gain and tibia zinc were the response variables. Additions of a zinc isotope to test materials as a tracer may augment the precision of a bioavailability estimation (Stuart et al., 1986).

In general, the addition of ^{65}Zn to natural products or diets is absorbed and retained to the same extent as that physiologically incorporated into the product, i.e., intrinsically labeled. Tibia

zinc concentration in rats fed labeled soy flour was less than that of rats fed reference zinc, but the specific activity of the zinc was not different. This lends credence to the use of isotope retention as a measure of absorption. Evans and Johnson (1977) first compared food extrinsically and intrinsically labeled with ^{65}Zn and found no difference in bioavailablility to the rat.

1.5.6. Metallothionein (MT)

MT is a low-molecular weight, thiol-rich, metal-binding protein, which was first identified as a Cd binding protein and was later shown to be a Zn and Cu binding protein. MT naturally binds seven gram atoms of divalent ions such as Zn, Cu and Cd. A molecule of MT is composed of two domains. The α-domain binds four atoms of metals, usually Zn, while the β-domain binds three atoms, usually Cu (Funk et al., 1987; Chang and Huang, 1996). It is well known that MT functions in absorption, metabolism, homeostasis and storage of both essential and non-essential trace metals (WHO, 1992; Chang and Huang, 1996; Kelly et al., 1996; Peraza et al., 1998). Many authors have shown that MT plays an important protective role in the prevention of toxicity from heavy metals such as Cd and Hg by formation of metal–MT combinations (Liu et al., 1992, 1994; Klaassen et al., 1999).

However, the main function of MT is maintenance of free metal Zn^{2+} and Cu^{2+} ions in the cells by acting as a homeostatic regulator and reservoir of Zn^{2+} (and Cu^{2+}) ions and as a donor of Zn for Zn-dependent biological processes. MT serves as a means of sequestering excess Zn as well as a Zn reservoir that can be utilized when Zn is deficient, releasing the metal when the organism is in a need of Zn for various cellular processes (Whanger and Ridlington, 1982; Petering et al., 1984; Kelly et al., 1996). MT can protect not only against Zn deficiency but also against its toxicity (Kelly et al., 1996). It has been proposed that during Zn overload, MT formed in the intestine binds Zn and thereby prevents further absorption. Because of its higher affinity to MT, Cd displaces Zn from the cysteine binding sites on this protein. As mentioned before, the increasing concentration of free Zn^{2+} ions may further induce synthesis of new molecules of MT (Jacobson and Turner, 1980; Petering et al., 1984; Funk et al., 1987; Chang and Huang, 1996). By displacing Zn, Cd interferes with Zn absorption, distribution into tissues and transport into cells or several intracellular structures and may inhibit its activities at various stages.

Many toxic effects of Cd occur through a disruption of Zn-mediated or Zn-dependent metabolic processes, including cellular production of DNA, RNA and protein (Sunderman and Barber, 1988). Disturbances in Zn function and metabolism, independently of cause, may have serious consequences for health. This element plays an important role in growth, development and functioning of all living cells (Bray and Bettger, 1990). It is involved as a co-factor in number of metalloenzymes (over 200) and regulatory proteins, including enzymes of both DNA and RNA biosynthesis and repair. The principal mechanism of the function of this trace bioelement is the modulation of activity of enzymes participating in replication, transcription and translation processes. By influencing the activity of many enzymes, Zn regulates the overall metabolism of the organism (Sunderman and Barber, 1988; Bray and Bettger, 1990; Lohmann and Beyerrsmann, 1993).

1.5.7. Aim of the work

Recent studies have indicated that the treatment with Se (El-Sharaky et al., 2007) or Zn (Jacquillet et al., 2006) protects tissues against the toxicity of Cd. However, the co-effect of the two trace elements on the toxicity caused by Cd is not yet well studied. Indeed, we have found only one published work in this context (Xiao et al., 2002) which assessed the protective effect of Se and Zn on Cd-induced oxidative stress in the kidney of the rat; besides, Cd was administrated using the intraperitoneal route. This led us to wonder about the simultaneous administration of i) Cd and organic Se and ii) Cd, Zn, and organic Se by oral route, as it is the main mode of exposure to Cd in humans and animals.

Therefore, it was considered of interest to investigate whether the combined treatment with organic Se and Zn offers more beneficial effects than that provided by either of them alone in reversing Cd-induced oxidative stress in broilers orally exposed to Cd.

The first experiment was designed to assess firstly, whether organic Se administered at high doses could protect broilers from Cd toxicity by evaluating the effects on performance and broiler health and secondly, to investigate the interactions between organic Se, Cd and 15 other elements, either toxic or essential in broilers.For that reason, performance, health and

accumulation of Se and Cd in liver, kidney, breast and blood tissues of 4- and 6-week old broilers fed diets with low and high levels of Se and Cd was investigated followed by determination of the concentration of Se, Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn as well as their interactions in blood, liver, kidney and breast muscle tissues.

In a second experiment, performance and health of broilers as well as the accumulation of 17 elements and their correlations in various broiler tissues after simultaneous administration of the maximal allowed levels of organic Se and Zn against orally induced Cd toxicity were evaluated.

PART B

Experiment 1

2. The role of selenium in cadmium toxicity: Interactions with essential and toxic elements (Al-Waeli et al., 2012)

2.1. Abstract

1. The study was part of a project designed to investigate if organic Se can ameliorate the toxic effects of Cd. The aim of the present study was to investigate if Se and Cd addition to chickens' diet affect the accumulation of Se Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn in the tissues.

2. Three hundred one day-old, chickens (broilers) were randomly distributed in four dietary treatments with 5 replicate pens per treatment. In T1, chickens were fed a diet with 0.3 ppm added Se, as Se yeast, without added Cd. In T2, chickens were fed a diet with 0.3 ppm Se and 10 ppm Cd, as $CdCl_2$. In T3, chickens were fed a diet with 0.3 ppm Se and 100 ppm of Cd added and in T4 treatment, chickens were fed a diet with 3 ppm Se and 100 ppm Cd added. On days 28 and 42, two chickens per replicate pen were sacrificed for collection of whole blood, liver, kidney and breast muscle samples. Samples were analyzed by ICP-MS.

3. While low Cd level in the diet led only to an increase of Cd concentration in the examined tissues, addition of high levels of Cd increased the concentration of Cd, Cu, Sb and V and decreased that of Se and Fe. Addition of high Se levels did not significantly reduce Cd concentration.

4. After application of the statistical model more than 50 correlations were noted. Most notably, Cd was correlated with Se, Ca, Co, Cu, Mg and As while Se was correlated with Cd, Li and Mn.

5. The present study revealed several correlations between essential, probably essential and toxic metals illustrating the importance of the balance between pro-oxidants and antioxidants.

2. 2. Introduction

Cadmium (Cd) is a heavy metal and is regarded as an environmental contaminant. It occurs both naturally and from industrial and agricultural sources (EFSA, 2009). There is a considerable effort worldwide to reduce Cd discharge and increase Cd-free technology (Figueroa, 2010). The occurrence of Cd is mainly associated with that of zinc (Zn) and to a lesser extent with that of lead (Pb) and copper (Cu) since it is found in several, mainly Zn-rich, ores and is considered as an inescapable side product of the metallurgy of these metals (Martelli et al., 2006). Animals are exposed to Cd via feed, water and contaminated air. The presence of Cd in animal production is a problem for several countries and its concentration in manure and feed can reach, in some cases, up to 130 ppm (Li et al., 2010a).

Cadmium is absorbed from the gastrointestinal tract and the lung and is mainly accumulated in liver and kidney where it is bound to metallothionein (MT). When the amount of Cd in the body exceeds the binding capability of MT, the non-MT-bound Cd ions cause hepato- and nephrotoxicity (Satish Rao et al., 2009). One mechanism proposed is that Cd not bound to MT can induce free radicals and lipid peroxidation, which may in turn depress hepatic and renal functions (Galazyn-Sidorczuk et al., 2009). Under physiological conditions, a balance between the amount of free radicals generated in the body and the production of antioxidants exists (Surai, 2006). The antioxidant defense system is in turn affected by alteration of trace element balance since several trace elements, like Se, Cu, iron (Fe) and Zn, are integral part of various antioxidant enzymes. Previous studies in rats revealed that essential trace metals like Zn and Se may modify health risks from exposure to Cd (Jemai et al., 2007, Jihen et al., 2008).

More specifically, Cd toxicity in rats was reduced after simultaneous administration of Zn and inorganic Se with Cd (Jemai et al., 2010; Messaoudi et al., 2010). On the other hand, deficiency in essential elements, like calcium (Ca), Fe and Zn, can enhance the susceptibility to Cd and Pb toxicity (Goyer, 1997; Tandon et al., 1994). Toxic metals can disrupt trace element metabolism and indeed Cd toxicity affects Ca metabolism either by direct toxicity to bone or indirectly from renal toxicity (Goyer, 1997; Pappas et al., 2010). Most of the aforementioned studies were conducted in mammals using inorganic Se. Information on interactions between Cd, Se and other trace elements both toxic and essential in poultry is sparsely (Li et al, 2010b; Pappas et al., 2011).

The present study was part of a bigger project designed to assess whether organic Se administered at high doses could protect broilers from Cd toxicity. The aim of the present study was to investigate the interactions between organic Se, Cd and 15 other elements, both toxic and essential in broilers. Therefore, the concentration of Se, Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn as well as their interactions were assessed in blood, liver, kidney and breast muscle tissues of 4 and 6 week old broilers fed diets with low and high levels of Se and Cd.

2.3. Materials and methods

2.3.1. Animals, diets and design

In brief, three hundred (300), as hatched, day-old, Cobb 500 chickens (broilers) were used in total. The chickens were obtained from a commercial hatchery. There were five replicate pens (2m length X 1m width) of four dietary treatments namely T1, T2, T3 and T4, randomly allocated in the house. Pen was the experimental unit. There were 15 chickens per pen, 75 per treatment. In T1 treatment, chickens were fed a diet with 0.3 ppm of Se added without Cd. In T2, chickens were fed a diet with 0.3 ppm of Se and 10 ppm of Cd added, as $CdCl_2$ (Sigma-Aldrich, St Louis, MO, USA). In T3, chickens were fed a diet with 0.3 ppm Se and 100 ppm Cd added and in T4 treatment, chickens were fed a diet with 3 ppm Se and 100 ppm Cd added (Table 1). Supplemented Se was from a yeast source, Sel-Plex® (Alltech Inc., Nicholasville, KY, USA).

The duration of the experiment was 42 days with housing and care of chickens, conforming to the guidelines of the bioethics committee 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 10^{th} day of their life, a grower diet to the 20^{th} day and a finisher diet to the 42^{nd} day (Table 1). Feed and water were provided ad libitum. The lighting program was 23 hours of light and 1 hour of dark. Stocking density was according to EU legislation. At the end of the 28th and 42^{nd} day, two chickens per pen (10 per treatment) were individual weighted, sacrificed and liver, breast, kidney and blood samples were collected for

trace element analysis. Furthermore, liver and kidney organs were weighted to calculate the organ to body weight ratio.

2.3.2. Trace Element determination

Selenium, Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn were determined in samples using inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, Elan 9000, Perkin Elmer Life and Analytical Sciences Inc, Waltham, MA, USA) as described previously. The instrumental parameters of the equipment used were: nebuliser flow 0.775 l min^{-1}, vacuum pressure 1.5×10^{-5} Torr, lens voltage 950W, 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 prior to feeding and milled prior to analysis through a 1 mm sieve (Cyclotec, 1093 sample mill, Tecator, Höganäs, Sweden). Samples (1g) of wet tissue or feed were soaked in 10 ml concentrated HNO3 (65% w/v, Suprapur, Merck, Darmstadt, Germany). Prior to analysis, complete digestion of the samples was performed using a microwave digestion system (CEM, Mars X-Press, NC, USA).

The samples were heated in the microwave accelerated digestion system according to the following program: the power was ramped during 20 min from 100 W to 1200 W 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) prior to injection in the ICP-MS instrument. Standard solutions used for calibration curves were prepared from high purity standards (Multielement standard solution, Fluka Analytical, Sigma-Aldrich, St Louis, MO, USA).

The analytical procedure was validated using a Se recovery procedure (Georgiou and Kouparis, 1990) and two standard reference materials (NIST-RM 8414, bovine muscle powder and NIST-RM 1577c, bovine liver powder - LGC Standards; Promochem, Wesel, Germany). The Se recovery procedure was as follows: four samples from the same liver tissue (1 g each) were spiked with 250, 500 and 750 μl of Se standard solution of 4 ppm, 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 96-111%. In Table 2, the reference and detected values of the reference materials are presented, indicating the accuracy of the method.

2.4. Statistical analysis

The statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC, USA). The data were analyzed using a multivariate linear model where Se, Cd, Ca, Co, Cu, Fe, Li, Mg, Mn, Sb, As, Cr, Pb, Mo, Ni, V and Zn concentrations 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 Bartlett's test of sphericity and by calculating the pairwise Pearson correlations. Both methods indicated that the concentrations are indeed correlated, which also reflects biological plausibility. Hence, a multivariate analysis appeared to be necessary. Data were log transformation prior to analysis to ensure approximate normality. The response variable (Y) was approximately normally distributed as verified by employing both histograms and normality tests. Thus, using a multivariate linear model seemed like a reasonable approach. The model was:

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

where Y denotes Se, Cd, Ca, Co, Cu, Fe, Li, Mg, Mn, Sb, As, Cr, Pb, Mo, Ni, V and Zn concentrations.

A particular aspect of our analysis was the comparison of the element correlations before and after running the model. In this way, potential differences (or lack thereof) may be revealed, which could otherwise be confounded due to ignoring the factor influence. The statements of significance presented in this study were based on $P \leq 0.05$ unless otherwise stated. In the tables, the data are presented as the mean ± SE of each of the main effects in turn, pooled for all other

main effects. Because the variance ratio of the main effects was greater than the variance ratio of their interactions, the presentation of the main effects is meaningful and legitimate. Furthermore, in the results section all statistically significant interactions are presented. Organ weights were also tested statistically using body weight as a covariant to evaluate the effect of body weight on organ weight.

2.5. Results

2.5.1. Treatment, age and tissue effects on the concentration of 17 elements

In Table 3 the concentration of minerals and trace elements for each examined factor is presented. As the age of broilers increased from 28 to 42 days, the concentration of Cd ($P<0.001$), Fe ($P=0.022$), Li ($P=0.002$), As ($P=0.001$), Pb ($P=0.005$), and V ($P<0.001$) significantly increased while that of Sb ($P=0.001$) significantly decreased. In detail, Cd, Fe, Li, As, Pb, and V concentration in 42 d old broilers increased by 63.3%, 17.9%, 13.2%, 35.1%, 12.5% and 74.0% respectively compared to that of 28d old broilers. Antimony concentration significantly reduced by 23.3% as the age of broilers increased.

Dietary Se and Cd addition to the diet (data pooled for age and tissue effects to exacerbate treatment effects) significantly affected the concentration of Se ($P<0.001$), Cd ($P<0.001$), Cu ($P<0.001$), Fe ($P<0.001$), Li ($P=0.018$), Sb ($P<0.001$), As ($P<0.001$), Mo ($P=0.003$), V ($P<0.001$) and Zn ($P=0.020$). Comparison of the elements' concentration between T1 (no Cd added) and T2 treatment (10 ppm of added Cd) revealed that only the concentration of Cd increased (26 ± 4 vs. 2114 ± 474 ppb) while the concentration of all the other elements did not alter. However when 100 ppm of Cd added to the diet (T3), comparison of elements' concentration with T1 treatment (no Cd added) revealed an increase of the concentration not only for Cd (26 ± 4 vs. 31270 ± 5427 ppb) but also for Cu (1685 ± 209 vs. 2462 ± 347 ppb), Sb (100 ± 13 vs. 134 ± 10 ppb) and V (16 ± 2 vs. 34 ± 5 ppb) accompanied with a decrease of the concentration of Se (388 ± 38 vs. 281 ± 33 ppb) and Fe (109 ± 16 vs. 77 ± 15 ppm).

Comparison of T3 and T4 treatments (both of them had 100 ppm of Cd and furthermore T4 had 3 ppm of added Se) revealed that the concentration of Se (281 ± 33 vs. 1880 ± 186 ppb)

and As (9.7 ± 1.6 vs. 11.9 ± 1.4 ppb) increased while that of Sb (134 ± 10 vs. 111 ± 9 ppb) and Fe (77 ± 15 vs. 70 ± 14 ppm) decreased. The concentration of the other elements was unaffected (Table 3). Furthermore, regarding the effect of dietary treatments on the ratio of liver's weight to body weight it was revealed that this ratio did not differ between the treatments being 2 % of body weight. However, the ratio of kidney's weight to body weight was significantly higher in treatments 3 and 4 (0.20 % of body weight), not differing between them, compared to that of treatments 1 and 2 (0.12 % of body weight) indicating that the weight of kidney increased in broilers fed diets with excess Cd (data not shown).

Regarding the concentration of the elements in the four examined tissues (data pooled for treatment and age effects to exacerbate tissue effects, $P<0.001$), it was revealed that kidney was the organ that contained the highest concentration of Se, Cd, Co, Li, As, Mo, and V in comparison to the other 3 examined organs while, liver contained the highest concentration of Cu, Pb, Mn and Zn. Blood contained more Fe and Sb compared to the other three examined organs while, in breast muscle tissue, higher concentration of Mg, Cr and Ni was noted in comparison to the other 3 organs. The concentration of Cu, Mn, Cr and Zn in the liver did not differ of that of kidney indicating that both these organs contained high concentration of these elements in comparison to the other 2 examined organs.

The significant age x tissue interactions (data pooled for treatment effects) revealed that the effect of broilers' age on the concentration of Se ($P<0.001$), Cd ($P<0.001$), Ca ($P<0.001$), Co ($P<0.001$), Cu ($P<0.001$), Fe ($P<0.001$), Li ($P<0.001$), Mg ($P<0.001$), Sb ($P=0.022$), As ($P<0.001$), Mo ($P=0.002$), V ($P<0.001$) and Zn ($P=0.001$) was different in each one of the four examined tissues. More specifically, in blood, as the age of broilers increased the concentration of As, V and Li increased while that of Se, Cd, Co, Cu, Sb and Mg decreased. In liver, as the age of broilers increased the concentration of Cd, Co, Cu, Se, Fe and Zn increased while that of As, Sb, and Li decreased. Regarding the kidney tissue, as the age of broilers increased the concentration of As, Cd and V increased while that of all other elements did not differ. Finally, in breast muscle tissue, as the age of broilers increased less As, V, Ca and Mo was deposited in the tissue while the concentration of the remaining examined elements did not differ. The most notable of all aforementioned age x tissue interactions was that of As. In detail, As concentration in breast muscle tissue in 42 day old broilers (2.8 ± 0.1 ppb) was reduced by 54% compared to that of 28 day old broilers (6.1 ± 0.2 ppb; data not shown).

The significant treatment x age interactions (data pooled for tissue effects) revealed that the effect of age on the concentration of several elements (Se ($P<0.001$), Ca ($P=0.002$), Cu ($P=0.016$), Fe ($P=0.011$), Li ($P<0.001$), Sb ($P=0.002$), As ($P=0.001$), Ni ($P=0.019$) and V ($P=0.001$) was different among broilers fed the four dietary treatments. Most notably, Se concentration for broilers fed the T1 diet increased as the age for broilers increased (345.84 ± 49.99 vs.430.27 ± 57.67 ppb). This was not evident for broilers fed diets T2, T3 and T4. Arsenic concentration increased with the age only for broilers fed the T2 (5.01±0.63 vs.11.91±1.98 ppb) and T3 diet (7.64±2.54 vs.11.46±1.98 ppb). The concentration of Ni increased as the age of broilers increased only for broilers fed the T3 diet (29.24±6.97 vs. 50.22±11.98 ppb). Similarly, V concentration increased with age only for broilers fed the T2 (9.38±0.97 vs. 27.00±5.13 ppb) and T3 (20.21±5.68 vs. 47.35±7.46 ppb) diet.

The significant treatment x tissue interactions indicated that the differences on several elements' concentration, Se ($P<0.001$), Cd ($P<0.001$), Co ($P<0.001$), Cu ($P<0.001$), Fe ($P<0.001$), Li ($P<0.001$), Sb ($P=0.002$), As ($P=0.001$), Ni ($P=0.019$) and V ($P=0.001$), due to the different dietary treatments were not reflected in the same manner in the four examined tissues. Most notably, blood Se concentration, in descending order, for the four dietary treatments was T4>T2=T1>T3 and this pattern was also noted in the kidney and breast muscle tissue. However, in the liver the pattern noted was T4>T2=T1=T3.

Significant treatment x age x tissue interactions were noted only for the following elements Se ($P<0.001$), Cd ($P=0.001$), Cu ($P=0.04$), Li ($P<0.001$), As ($P<0.001$), Ni ($P=0.021$) and V ($P<0.001$) indicating that concentration in some treatments for some tissues responded different with increasing age. It is worth mentioning that at 28 d old broilers, Cu concentration in blood of broilers fed the T3 diet was higher than that of broilers fed the T4 diet while in 42 d old broilers, Cu concentration in blood of broilers fed the T3 diet did not differ from that of broilers fed the T4 diet.

2.5.2. Pre-and Post-model correlations

The correlations between the trace elements before and after the application of the model are presented in Table 4. After the application of the statistical model, the discernible points by inspecting the results were that Co, Zn and Mn were correlated with 9 other elements; Ca, Cu and Mg were correlated with 8 elements; Mo was correlated with 7 elements; Cd, Fe, Li, As and Ni were correlated with 6 elements, V with 5 elements; Cr with 4 ones; Se with 3; Sb with 2 and finally that Pb was not correlated with anyone. In addition, the value and significance of some pairwise correlations changed by incorporating the factors' influence.

Specifically, Cd was correlated with Se, Co, Cu, Mg, As and this relationship remained after the application of the model however, for As, this relationship became negative after model application. The seemingly strong correlation of Cd with Li, Mn, Pb, Mo, V and Zn was in fact spurious as the elements were in fact uncorrelated post-model. In contrast, Cd and Ca appeared uncorrelated in the descriptive statistics, while in fact they were found to correlate positively when the examined factors were taken into account. Hence, this association was masked by ignoring the considered factors. In the descriptive statistics, Se apart of its aforementioned correlation with Cd, appeared to be correlated positively with Ca, Co, Cu, Mn, As, Pb, Mo, V, Zn and negatively with Ni, but when the factors of the study were considered only its correlation with Mn was a significant but negative one. In contrast, the seemingly uncorrelated Se and Li were found to be weekly negatively correlated. Prior to application of the model, Ca was correlated with Co, Fe, Mg, Sb, As, Cr, Pb, Mo, and V but post-model it was correlated only with Co, Fe, Mg and Mo and furthermore with Cu, Mn and Zn, the latter 3 correlations appeared masked in the descriptive statistics.

Cobalt was correlated positively with Cu, Fe, Li, Mg, Mn, As, Pb, Mo, V, Zn and negatively with Ni however, some of this correlations were spurious since post-model it was positively correlated only with Cu, Mg, Mn, Mo, Ni, Zn and furthermore with Sb, the latter one being masked in the descriptive statistics. In the descriptive statistics, Cu was positively correlated with Li, Mg, Mn, As, Cr, Pb, Mo, V, Zn and negatively with Sb and Ni but after the application of the model, Cu was correlated only with Mg, Mo, Ni and Zn and furthermore with Fe. Iron was negatively correlated with Li, Mg, Cr and Ni and positively with Sb, As, Pb, Mo

and V but post-model was correlated positively with Mo, Ni and Cr. Furthermore, Fe was correlated with Zn, a correlation that was masked initially.

Lithium was positively correlated with Mg, Mn, As, Mo, V and Zn and these relationships remained so post-model, except for Mg. Regarding Mg, the application of the model revealed that the seemingly strong correlation of Mg with Sb, As and Pb was spurious as in fact Mg was uncorrelated with these elements. However, prior to the application of model, Mg was correlated negatively with V, and positively with Mn, Cr and Zn. Post-model, Mg was positively correlated with all four. Manganese, prior to the application of the model, was positively correlated with As, Cr, Pb, Mo, V and Zn and negatively with Sb and Ni. After the application of the model, these relationships remained so except for those with Sb, Pb, Mo and Zn. It should be noted that the post-model correlation of Mn with Ni became a strong positive one.

Antimony seemed to be correlated with Cr (negatively), V and Zn (negatively) but after the application of the model was only correlated with V (negatively). In the descriptive statistics, As was correlated to Cr (negatively), Mo, Ni (negatively) and V, but after the application of the model it was positively correlated only with Mo and V and furthermore with Zn, a seemed masked correlation prior to the application of the model. Initially, Cr was correlated with Ni, V (negatively), and Zn but remained correlated only with Ni. It should be mentioned that consideration of the examined factors of the model revealed that Pb was not correlated with any one of the other 16 elements, although spuriously seemed to be correlated with 8 of them. Molybdenum was correlated with Ni (negatively), V and Zn but remained correlated only with Zn. Nickel was negatively correlated with Zn but in fact the two elements were uncorrelated and finally, prior and after the application of the model V and Zn were correlated.

2.6. Discussion

The aim of the present study was to investigate whether organic Se administered at high doses could protect broilers from Cd toxicity. It was hypothesized that high Se added levels, well below toxic ones (Gad and Abd El-Twab, 2009) could maintain the antioxidant-pro-oxidant balance and reduce the adverse effects of Cd. However, solely by examining the levels of deposited Cd in the tissues, this was not affirmed. Selenomethionine, a major constituent of Se-yeast, is incorporated non-specifically into proteins in place of methionine (Rayman, 2004) and it is believed that stored tissue Se levels can be mobilized and used in cases of oxidative stress. In our previous work (Pappas et al, 2011), it was shown that organic Se added at several concentrations, from 0.15 up to 3 ppm, could reduce the tissue deposition of the low Cd levels (no added Cd, only that present in the diet). It is possible that the discrepancy of results reported in the present study and our previous work is related to the ratio of Se/Cd. It is possible that 3 ppm of added Se could not ameliorate all the toxic effects of 100 ppm of added Cd. When Se is administered as a remedy for heavy metal toxicity, it should form insoluble compounds to be excreted in the faeces. Complexes of heavy metals with organic and inorganic sources of Se exhibit different solubility (Feroci et al., 2005). Although direct comparison with inorganic Se cannot be made since it was not used in the present study, previous work with inorganic Se indicated that 0.1 ppm Se, as Na_2SeO_3, added to diets contain 200 ppm of $CdCl_2$ did not induce any significant change in the levels of Cd accumulation in rat's kidney, liver (Jihen et al., 2008) and testis (Messaoudi et al., 2010).

However, inorganic Se supplementation reduced Cd levels in the liver and kidney of rat pups (Lazarus et al., 2009a). These authors administered orally to suckling rats equimolar doses of Cd and Se. It is possible that differences regarding reduction or not of Cd accumulation by Se noted between different studies may be related with the administered doses, the mode of application, and the duration of exposure. Previous work with in vitro produced selenite and sulfide (Na_2S) revealed that transition metal ions, like Cd^{2+}, could form a complex, which then bounds to the plasma protein selenoprotein P to form a ternary complex (Sasakura and Suzuki, 1998) indicating that selenoprotein P may be also involved during formation of complexes with Cd.

The present study revealed that the level of Cd present the diet determines the accumulation of other elements namely Cu, Fe, Sb, Se and V. While low levels of added Cd only affected the level of accumulated Cd, high levels of added Cd increased the concentration of Cd, Sb, V and reduced that of Se and Fe. As mentioned in the introduction, absorbed Cd is preferably accumulated in kidney and liver bound to MT. Metallothionein synthesis is induced by monovalent silver, divalent Cd, Zn, Cu and Hg, trivalent bismuth compounds and trivalent cerium (Kobayashi et al., 2006). When Cd concentration exceeds the binding capacity of MT, the non-bound Cd causes toxicity possibly due to induction of oxidative stress, inhibition of DNA repair, deregulation of cell proliferation (Sarkar et al. 1997; Beyersmann and Hartwig, 2008) and DNA methylation (Zhang et al., 2009).

It is possible that the reduction of accumulated Se in the tissues is related with its use either for the formation of Se-Cd complexes (El-Sharaky et al., 2007) or for antioxidant protection against the induced oxidation by Cd (Surai, 2006). Similarly, Fe is involved in the antioxidant defense system of the organism via the action of catalase which is a tetrameric heme-containing enzyme, located primarily in the peroxisomes (Zoidis et al., 2010). Catalase detoxifies H_2O_2 by catalyzing its breakdown to water and divalent oxygen. It is possible that the reduction of deposited Fe by high levels of Cd present in the diets is linked with the antioxidant protection provided by catalase. Although the mechanisms underlying the interaction of Sb and Cd are still obscure, Sb is known to react with sulfhydryl groups of proteins, acting as an enzyme inhibitor (Beyersmann and Hartwig, 2008) and exerting toxic effects (Jarzyńska, and Falandysz, 2011).

Vanadium has been reported to stimulate immunological responses of chicks by inhibiting protein-tyrosine-phosphatase thus increasing protein-tyrosine-phosphate levels in macrophages which could in turn affect immunity (Qureshi et al., 1999). Another important physiological role of V is that it mimics the action of insulin (Crans et al., 2004). On the other hand, V appears to exert its toxic effect through inhibition of enzymes and cell damage (Henry and Miles, 2001). The vanadyl cation behaves like a simple divalent ion which can compete for ligand binding sites with Ca^{+2}, Mn^{+2}, Zn^{+2}, and Fe^{+2}. It is possible that Cd and V are linked via the action of MT since recently it was shown in mice that a pentavalent vanadium compound can induce MT synthesis (Kobayashi et al., 2006).

Addition of high Se levels to diets containing high Cd levels led to a decrease of Sb and Fe concentration and to an increase of As concentration. It is possible that Se and Fe are linked

via the action of the non selenoenzyme catalase. Zoidis and co-workers (2010) showed that liver catalase mRNA levels of 4 wk old broilers were significantly decreased as Se supplementation increased. The same authors reported that it was not clear whether the reserves built by excess of Se surplussed the needs for antioxidant protection provided by catalase, thus no additional catalase needed to be transcribed or that other factors influenced the relation between Se status and catalase. It seems that Se status under certain conditions, i.e. age (Semsei et al., 1991), vitamin availability (Avanzo et al., 2002) and environmental conditions such as cold stress (Yuksel et al., 2008), influences both gene expression and activity of catalase. The reduction of Sb concentration and the increase of As concentration when high levels of Se are added to the diet, containing high levels of Cd, may be related with the formation of glutathione (GSH). It has been reported that As and Sb induce dramatic increases in the biliary excretion of GSH because they are excreted in bile as complexes with GSH leading to partial depletion of this thiol from the liver. Since selenols are chemically similar to thiols, it is possible that they also react with Sb and As (Gregus et al., 1998).

The current study revealed more than 50, post model, correlations between elements. A significant, post model, interaction between two elements indicates that above and beyond the examined factors of the study a correlation exists between the two elements. Most notably, the Se-Cd correlation was a weak positive one. Strong positive Se-Cd correlation has been reported in the liver of dolphins (Lavery et al., 2008). In contrast, negative correlation between Se and Cd has been reported in other studies with cattle (López Alonso et al., 2004) or broilers (Pappas et al., 2011). Other authors reported no correlation between Se and Cd in cattle (García-Vaquero et al., 2011). Correlations between elements presented in different studies are not directly comparable due to the different design of each study.

Furthermore, it’s not clear whether these differences are related to the ratio of Cd and Se present in the diet, to the formation of complexes or other indirect interrelations that need to be elucidated. The negative correlation between Li and Se may be attributed to the effects that these elements have on thyroid hormone. Lithium is a potent inhibitor of thyroid hormone release (Lazarus et al., 2009b) and in humans, urinary Li was negatively correlated to plasma free thyroxine (T4) while urinary Se was positively correlated with free T4 (Broberg et al., 2011). In the present work, a positive Cd-Ca correlation was reported. The results are in line with results from other studies (López Alonso et al., 2004).

Cadmium ions have ionic radii very similar to those of Ca^{2+} and even though the preferred ligand of Ca^{2+} is oxygen, whereas of Cd^{2+} is sulfur, Cd^{2+} can accept oxygen and substitute Ca^{2+} in protein binding sites (Beyersmann and Hartwig, 2008). Cadmium can increase the intracellular concentration of Ca by promoting Ca efflux from the sarcoplasmic reticulum (Martelli et al., 2006). The Cd-Co and Cd-Cu correlations reported in the present study corroborate previous reports in cattle (López Alonso et al., 2004) and healthy humans (Whitfield et al., 2010), respectively. Cadmium is able to generate indirectly various radicals like the superoxide radical. These radicals are scavenged by GSH. Although cobalt's main known function is to act as an integral part of vitamin B12, electron paramagnetic resonance spectroscopy studies with Co revealed that GSH can be switched from antioxidant to pro-oxidant indicating that Co species may cause cellular damage under suitable conditions (Valko et al., 2005). The indirect role of Cd in free radical generation may be related to the replacement of Fe and Cu in several cytoplasmic and membrane proteins which leads to increase the amount of unbound free or chelated Cu and Fe ions participating in oxidative stress via Fenton reactions (Valko et al., 2005).

In the present study, Zn was found not be correlated with Cd. Zinc is an integral part of the enzyme CuZn-superoxide dismutase (CuZn-SOD) and it is involved in cell membrane stabilization and MT synthesis (Schinina et al., 1996). Zinc supply in conditions of exposure to Cd can partially protect against Cd-induced oxidative stress and restore CuZn-SOD activity in red blood cells of rats (Jemai et al., 2007) while Se and Zn can have a cooperative effect in the protection against Cd-induced structural damage in the liver but not in the kidney of rats (Jihen et al., 2008).

In conclusion, the present study revealed several correlations between essential, probably essential and toxic metals indicating the importance of the balance between pro-oxidants and antioxidants. Trace element correlations reported in this study do not necessarily indicate that the level of essential elements, like Se, can determine the amount of toxic metal accumulation in broiler tissues. Interpretation of in vivo results is not easy since trace elements may interact at the levels of absorption, distribution and retention.

Table1. Composition (g kg^{-1}), calculated analysis and determined Se and Cd concentration (ppb) of the experimental broiler diets

Ingredients (g kg^{-1})	**Starter (0-10 d)**	**Grower (11-20 d)**	**Finisher (21-42 d)**
Maize	559.5	631.0	658.4
Soybean meal	333.7	261.0	226.4
Gluten	50.0	50.0	50.0
Soybean oil	15.4	16.9	25.7
Dicalcium phosphate	15.1	14.4	13.2
Limestone	16.1	15.7	14.8
Lysine	0.2	1.5	2.1
Methionine	1.1	1.3	1.5
NaCl	4.9	4.2	3.9
Premix[1]	4.0	4.0	4.0
Calculated Analysis			
ME (MJ kg^{-1})	12.5	12.9	13.3
CP (g kg^{-1})	210.0	190.0	180.0
Sodium (g kg^{-1})	2.0	1.7	1.6
Ca (g kg^{-1})	10.0	9.6	9.0
Available P (g kg^{-1})	5.0	4.8	4.5
Methionine	5.3	5.1	5.2
Methionine+ cysteine (g kg^{-1})	8.9	8.4	8.2
Lysine (g kg^{-1})	12.0	11.0	10.5

Treatment	**Se added[2] (ppb)**	**Se determined[3] (ppb)**	**Cd added[4] (ppb)**	**Cd determined[3] (ppb)**
T1	300	459 ± 23	0	175 ± 15
T2	300	451 ± 14	10,000	11,923 ± 1564
T3	300	456 ± 11	100,000	105,877 ± 3189
T4	3000	3570 ± 100	100,000	101,017 ± 421

[1]Premix supplied per kg of diet: 12,000 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, 0.025 mg 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.

[2]Se was added in the form of Sel-Plex® (Alltech Inc, Nicholasville, KY, USA)

[3]The determined Se and Cd contents represent pooled data from all three diets (starter, grower and finisher)

[4]Cd was added in the form of $CdCl_2$

Table2. Determined and certified trace element concentration in the reference material NIST-RM 8414 (bovine muscle powder) and NIST-RM 1577c (bovine liver powder)

Element	RM 8414			RM 1577c		
	Detected value (ppb)	**Certified value (ppb)**	**Recovery (%)**	**Detected value (ppb)**	**Certified value (ppb)**	**Recovery (%)**
Se	71±10	76±10	93	2437±194	2031±45	120
Cd	14± 3	13±11	109	114±6	97±1	118
Ca	132,366±5388	145,000±20,000	91	125,859±5332	131,000±10,000	96
Co	8±0.44	7± 3	114	362±18	300±18	121
Cu	2735±43	2840±450	96	262,392±16,807	275,200±4600	95
Fe	73,506±1769	71,200±9200	103	237,651±12,182	197,940±650	120
Li	18±2	-	*NA*	19±3	12	158
Mg	1,025,599±12,137	960,000±95,000	107	687,602±45,443	620,000±42,000	111
Mn	367±11	370±90	99	12,225±633	10,460±470	117
Sb	8±5	10	80	5±4	3±0	158
As	12±2	9± 3	133	27±1	19± 1	142
Cr	66±1	71±38	93	70±3	53±14	132
Pb	405±20	380±240	107	80±6	62± 1	129
Mo	91±4	80±60	114	4002±200	3300±130	121
Ni	52±3	50±40	104	61±4	44±9	139
V	8±4	8± 0	100	7±2	5	140
Zn	130,725±1511	142,000±14,000	92	177,470±12,071	181,100±1000	98

Table3. The effect of Se and Cd supplementation in the diet of broilers on the concentration (ppb) of 17 elements in blood, liver, kidney and breast tissue at 28 and 42 days of age

Element	Factor Studied										P Value						
	Age (d)		Treatment				Tissue				Source of Variation						
	28	42	T1	T2	T3	T4	Blood	Liver	Kidney	Breast	Age	Treat	Tissue	Treat X Age	Treat X Tissue	Age X Tissue	Treat X Age X Tissue
Se	665 ±83	795 ±117	388[a] ±38	373[a] ±42	281[b] ±33	1880[c] ±186	295[a] ±38	934[b] ±104	1301[c] ±217	393[d] ±87	0.403	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Cd	11928[a] ±2375	19474[b] ±3657	26[a] ±4	2114[b] ±474	31270[c] ±5427	29393[c] ±5172	183[a] ±38	27769[b] ±5149	34625[c] ±517	228[a] ±36	< 0.001	< 0.001	< 0.001	0.689	< 0.001	< 0.001	0.001
Ca	38337 ±2629	35083 ±2134	41709 ±4473	34919 ±3281	32462 ±2582	37750 ±2840	56457[a] ±3253	23939[b] ±982	49115[a] ±1642	17329[c] ±2173	0.106	0.091	< 0.001	0.002	0.213	< 0.001	0.389
Co	27.43 ±2.00	31.38 ±2.52	29.07 ±3.31	29.97 ±3.50	30.10 ±3.13	28.47 ±3.05	15.01[a] ±0.97	35.62[b] ±1.66	57.29[c] ±1.54	9.70[d] ±0.49	0.300	0.544	< 0.001	0.533	< 0.001	< 0.001	0.844
Cu	1977 ±186	2187 ±225	1685[a] ±209	1750[a] ±223	2462[b] ±347	2429[b] ±347	385[a] ±31	3807[b] ±147	3710[b] ±191	425[c] ±11	0.100	< 0.001	< 0.001	0.016	< 0.001	< 0.001	0.040
Fe	85249[a] ±11018	100512[b] ±11782	109056[a] ±16831	114561[a] ±17500	77463[b] ±15263	70442[c] ±14137	251308[a] ±5498	77974[b] ±9732	38370[c] ±2554	3869[d] ±182	0.022	< 0.001	< 0.001	0.011	< 0.001	< 0.001	0.421
Li	11.94[a] ±1.45	13.52[b] ±1.13	10.08[a] ±1.34	12.27[ab] ±1.520	14.62[ab] ±2.39	13.96[b] ±1.92	10.41[a] ±1.89	7.01[a] ±0.45	23.58[b] ±2.40	9.92[c] ±0.31	0.002	0.018	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Mg	196516 ±10492	197783 ±11232	193135 ±14319	197099 ±15183	199501 ±16065	198864 ±16213	50130[a] ±1279	217379[b] ±2513	203601[c] ±1936	317489[d] ±2883	0.127	0.893	< 0.001	0.826	< 0.001	< 0.001	0.199
Mn	1362 ±139	1363 ±148	1475 ±217	1566 ±236	1222 ±185	1198 ±173	122[a] ±21	2776[b] ±82	2359[b] ±62	132[c] ±7	0.121	0.077	< 0.001	0.331	< 0.001	0.112	0.307
Sb	127[a] ±9	103[b] ±6	100[a] ±13	115[ac] ±12	134[b] ±10	111[c] ±9	193[a] ±10	84[b] ±9	111[c] ±7	72[b] ±4	0.001	< 0.001	< 0.001	0.002	0.001	0.022	0.089
As	7.87[a] ±0.87	10.63[b] ±0.92	6.87[ab] ±0.84	8.68[ab] ±1.17	9.70[a] ±1.59	11.91[b] ±1.38	13.34[a] ±1.75	6.28[a] ±0.32	13.53[b] ±1.45	4.40[c] ±0.28	0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001
Cr	23.38 ±0.63	23.10 ±0.60	22.65 ±0.75	23.44 ±0.95	24.54 ±1.00	22.34 ±0.71	19.58[a] ±0.93	25.40[b] ±0.47	22.31[b] ±0.52	25.67[b] ±1.02	0.711	0.225	< 0.001	0.104	0.870	0.255	0.132
Pb	9.71[a] ±0.88	10.92[b] ±0.62	8.82 ±0.83	10.69 ±1.26	10.14 ±1.00	11.61 ±1.17	11.39[ab] ±0.94	14.08[a] ±1.26	9.41[b] ±0.93	6.38[c] ±0.78	0.005	0.053	< 0.001	0.322	0.240	0.145	0.424
Mo	359.8 ±34.0	359.4 ±33.39	379[ab] ±51	392[a] ±50	338[ab] ±45	329[b] ±45	146[a] ±20	554[b] ±17	712[c] ±20	27[d] ±2	0.055	0.003	< 0.001	0.701	0.096	0.002	0.739
Ni	27.87 ±2.33	31.85 ±3.48	27.43 ±2.68	21.88 ±1.78	39.73 ±7.04	30.39 ±2.68	31.32[ab] ±3.23	23.42[a] ±2.89	21.93[a] ±1.50	42.76[b] ±6.59	0.362	0.051	< 0.001	0.019	0.006	0.072	0.021
V	19.49[a] ±2.52	33.92[b] ±3.19	16.38[a] ±2.41	18.44[a] ±3.04	33.78[b] ±5.11	37.37[b] ±4.55	30.50[ab] ±3.41	20.76[a] ±2.44	49.32[b] ±5.66	7.14[c] ±0.72	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001
Zn	12931 ±1007	14441 ±1218	14638[a] ±1736	14579[ab] ±1614	12863[ab] ±1528	12665[b] ±1465	5312[a] ±1321	23321[b] ±839	20937[b] ±585	5176[c] ±116	0.093	0.020	< 0.001	0.728	0.130	0.001	0.074

Data were transformed (log transformation) prior to analysis. Values are the non transformed means ± SEM (n=5) of the main effects, pooled for all other main effects based on Multivariate Regression. Means within a row, within a main effect comparison, with different superscripts are different at $P<0.05$, unless otherwise stated. Statistical differences indicated in the table and P values refer to transformed data.

Table 4. Correlations between the concentrations of 17 elements prior to and after the application of the statistical model

	Cd	Ca	Co	Cu	Fe	Li	Mg	Mn	Sb	As	Cr	Pb	Mo	Ni	V	Zn
Prior to model application																
Se	0.602***	0.249**	0.603***	0.645***	0.108	0.122	0.142	0.611***	-0.102	0.251**	0.010	0.289***	0.555***	-0.158*	0.353***	0.559***
Cd		0.129	0.639***	0.697***	-0.046	0.258**	0.231**	0.585***	0.081	0.205*	0.138	0.286***	0.532***	-0.087	0.427***	0.559***
Ca			0.400***	0.124	0.662***	0.070	-0.616***	0.119	0.453***	0.349***	-0.419***	0.268**	0.473***	-0.039	0.498***	0.097
Co				0.865***	0.267**	0.229**	0.164*	0.845***	0.060	0.192*	0.098	0.290***	0.877***	-0.169*	0.349***	0.821***
Cu					0.067	0.221**	0.414***	0.943***	-0.231**	0.169*	0.244**	0.251**	0.826***	-0.267**	0.284***	0.937***
Fe						-0.264**	-0.814***	0.077	0.436***	0.227**	-0.333***	0.385***	0.517***	-0.164*	0.292***	0.035
Li							0.241**	0.224**	-0.084	0.631***	-0.014	-0.079	0.186*	-0.090	0.496***	0.210**
Mg								0.433***	-0.625***	-0.225**	0.512***	-0.209**	-0.063	0.031	-0.284***	0.470***
Mn									-0.283***	0.167*	0.267**	0.285***	0.804***	-0.232**	0.255**	0.931***
Sb										0.035	-0.266**	0.117	0.049	0.096	0.183*	-0.324***
As											-0.213**	0.121	0.331***	-0.184*	0.753***	0.142
Cr												0.003	-0.016	0.286***	-0.232**	0.288***
Pb													0.346***	0.008	0.298***	0.236**
Mo														-0.353**	0.428**	0.777**
Ni															-0.115	-0.241**
V																0.222**
Post model application																
Se	0.168*	-0.047	-0.059	0.064	0.112	-0.171*	-0.016	-0.250***	-0.045	-0.101	-0.062	0.065	-0.086	-0.069	-0.017	0.060
Cd		0.183*	0.281***	0.290***	0.131	0.028	0.332***	0.033	0.031	-0.193*	0.076	0.025	0.020	0.158	-0.000	0.093
Ca			0.241**	0.337***	0.169*	0.113	0.262***	0.277***	-0.053	0.096	-0.081	0.132	0.177**	0.111	0.147	0.419***
Co				0.291***	0.026	0.126	0.411***	0.253***	0.452***	0.027	0.064	0.045	0.245***	0.246***	0.025	0.246***
Cu					0.201*	0.102	0.407***	0.101	0.002	-0.009	0.131	0.064	0.214**	0.189**	-0.008	0.607***
Fe						0.158	0.135	0.088	0.046	0.124	0.195**	0.094	0.211**	0.191**	0.046	0.181**
Li							0.047	0.340***	-0.072	0.628***	0.020	-0.090	0.336***	0.040	0.595 ***	0.220**
Mg								0.329***	0.140	-0.105	0.295***	0.097	0.142**	0.287***	-0.095	0.391***
Mn									0.040	0.226**	0.217**	0.097	0.147	0.245***	0.201**	0.153
Sb										-0.073	0.086	0.016	0.078	0.101	-0.221**	-0.028
As											-0.048	0.019	0.262***	-0.016	0.708***	0.211**
Cr												0.160	0.106	0.358***	-0.044	0.093
Pb													-0.083	0.141	-0.009	0.104
Mo														0.036	0.047	0.332***
Ni															0.054	0.108
V																0.198**

Notes: Level of statistical significance of each of the pairwise correlations, *P≤0.05; **P≤0.01; ***P≤0.001

3. The role of selenium in cadmium toxicity: Effects on broiler performance and health status (Al-Waeli et al., 2013)

3.1. Abstract.

1. This work was part of a project designed to assess if organic selenium (Se) can protect against the toxic effects of cadmium (Cd).

2. A total of 300 day-old, as hatched, broilers were randomly distributed in four dietary treatments with 5 replicate pens per treatment. In T1 treatment, chickens were fed a diet with 0.3 ppm added Se, as Se yeast, without added Cd. In T2, chickens were fed a diet with 0.3 ppm Se and 10 ppm Cd, as $CdCl_2$. In T3, chickens were fed a diet with 0.3 ppm Se and 100 ppm of Cd added and in T4 treatment, chickens were fed a diet with 3 ppm Se and 100 ppm Cd added. On the 4^{th} and 6^{th} week, two broilers per replicate pen were sacrificed for whole blood, liver, kidney and breast samples. Body mass, feed conversion ratio and mortality were assessed and hematological analyses were performed. Se and Cd levels in tissues were analyzed by inductively coupled plasma mass spectrometry.

3. Low levels of Cd added to the diets had no significant negative effect on performance, while addition of excess Cd to the diet led to an impairment of broilers' performance. The examined hematological parameters ranged within physiological values revealing no negative health effects after simultaneous Cd and Se addition.

4. The present study indicated that Se can help against the negative effects of Cd but cannot counteract all of its negative effects.

3.2. Introduction

Trace elements are required in minute quantities for the proper growth, development and physiology of the organism. They can be classified as (i) essential, e.g. iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and selenium (Se), (ii) probably essential, e.g. cobalt (Co), nickel (Ni) and vanadium (V), and (iii) potentially toxic, including but not limited to aluminium (Al), arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg). Essential and probably essential elements are required in the diet but that may have toxic effects at supranutritional concentrations (Underwood and Suttle, 1999). On the contrary, potentially toxic ones are not required in the diet and cause chronic negative effects at low concentrations and lethal effects at high concentrations (Bires et al., 1995; Uluozlu et al., 2009).

Cadmium is known for its toxicity and the International Agency for Research on Cancer (IARC) has classified Cd as a Category I carcinogen (IARC, 1993). To ensure animal and human health, EU recommends that Cd concentration in feed should remain below 5 mg per kg of feed (EC, 2005). Furthermore, EU specifies that, feed materials of vegetable origin should contain less than 1 ppm, feed materials of animal origin less than 2 ppm and additives belonging to the functional group of compounds of trace elements less than 10 ppm with the exception of copper oxide, manganous oxide, zinc oxide and manganous sulphate monohydrate that should contain less than 30 ppm. The maximum limit for Cd in water is 5 ppb (EC, 1998).

International green trade barriers for agricultural products and domestic feed safety issues, combined with public awareness on the impact of Cd pollution led to a considerable worldwide effort to reduce Cd discharge (Li et al., 2010b). Currently, Cd's occurrence in food is a major issue for public health and in 2011; the panel on contaminants in the food chain of the European Food Safety Authority (EFSA) issued a statement maintaining the tolerable weekly intake at 2.5 μg per kg of body weight (EFSA, 2011).

Cadmium is an inescapable side product of the metallurgy of several metals like Zn (Martelli et al., 2006) and in some parts of the world, Cd presence in feed (Li et al., 2010b) and manure (Liu et al., 2005) can exceed imposed limits. Several studies investigated ways to reduce Cd toxicity by administering other elements as a remedy (Groten et al., 1991; Jemai et al., 2007; Jihen et al., 2008) since absorption, accumulation and toxicity of a trace element may be affected by a

variety of factors, including element's level in feed ingredients, its chemical form, ability to form complexes and interactions with other elements (Valko et al., 2005; Lopez-Alonso et al., 2007; Pappas et al., 2010). Selenium is a trace element known to play a pivotal role in the antioxidant defence system protecting the organism form oxidative stress (Pappas et al., 2008; Zoidis et al., 2010). The majority of studies examining ways to reduce Cd toxicity with Se were conducted in mammals using inorganic Se (Jihen et al., 2008; Messaoudi et al., 2010). Selenium-yeast is a highly available form of Se for livestock and provides antioxidant protection at a level greater than inorganic selenium (Mahan, 1999; Mahmoud and Edens, 2003).

The present study was part of an environmental project designed to assess the effects of contamination of broilers' feed with heavy metals on performance and accumulation of toxic elements in their tissues. An important aspect of the project was to evaluate the effects of Cd toxicity and if they can be ameliorated by the presence of organic Se. Therefore, performance, health and accumulation of Se and Cd in liver, kidney, breast and blood tissues of 4- and 6-week old broilers fed diets with low and high levels of Se and Cd was investigated.

3.3. Materials and Methods

3.3.1 Animals, diets and design

The study was designed considering that (1) the EU limit of Cd in feed is 5 ppm (EC, 2005), (2) in some cases, levels of Cd in feed can exceed maximum permitted limits (Li et al., 2010b), (3) the addition of elevated levels of essential elements may ameliorate the negative effects of toxic metal contamination (Nolan and Brown 2000; Jemai et al., 2007; Jihen et al., 2008), and (4) the maximum allowed Se inclusion level in the US is 0.3 ppm (Payne et al., 2005).

Three hundred (300), as hatched, day-old, Cobb 500 broilers were used in total. The broilers were obtained from a commercial hatchery. There were five replicate pens of four dietary treatments namely T1, T2, T3 and T4, randomly allocated in the house. Pen was the experimental unit. Each replicate was assigned to a clean concrete floor pen (2 m^2) and birds were raised on a wheat straw shavings litter. There were 15 broilers per pen, 75 per treatment. In T1 treatment, broilers were fed a diet with 0.3 ppm Se added without Cd. In T2, broilers were fed a diet with 0.3 ppm Se and 10 ppm Cd added as $CdCl_2$ (Sigma-Aldrich, St Louis, MO, USA). In T3, broilers were

fed a diet with 0.3 ppm Se and 100 ppm Cd added and in T4 treatment, broilers were fed a diet with 3 ppm Se and 100 ppm Cd added (Table 5). Supplemented Se was from a yeast source, Sel-Plex® (Alltech Inc., Nicholasville, KY, USA).

The duration of the experiment was 42 days with housing and care of broilers, conforming to the guidelines of the bioethics committee Faculty of Animal Science and Aquaculture of the Agricultural University of Athens. The broilers were raised, according to Cobb's management manual, in a house where light and ventilation were controlled. The lighting program was 23 hours of light and 1 hour of dark. Heat was provided with a heating lamp per pen. The broilers were fed a starter diet to the 10th day of their life, a grower diet to the 20^{th} day and a finisher diet to the 42nd day (Table 5). Feed and water were provided *ad libitum*. Stocking density was according to EU legislation.

At weekly intervals, broilers were weighted, their body mass recorded and the weekly mean body mass gain (WMBG) calculated. Furthermore, feed intake was measured weekly and weekly mean feed consumption (WMFC), and feed to gain ratio (FCR) were calculated. At the end of the 28^{th} and 42^{nd} day, two broilers per pen were sacrificed so that liver, breast, kidney and blood samples could be collected for Se and Cd determination. Broilers were inspected daily and mortality was recorded on the appropriate data capture form. Total mortality was calculated as the number of broilers that died throughout the study compared to the initial number of broilers placed corrected for broilers removed for blood collection.

3.3.2. Selenium and Cd determination

Selenium and Cd were determined in feed and tissue samples using inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, Elan 9000, Perkin Elmer Life and Analytical Sciences Inc, Waltham, MA, USA) (Pappas et al., 2011). The instrumental parameters of the equipment used were: nebulizer flow 0.775 l min^{-1}, vacuum pressure 1.5×10^{-5} Torr, lens voltage 950W, 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 prior to feeding and milled prior to analysis through a 1 mm sieve (Cyclotec, 1093 sample mill, Tecator, Höganäs, Sweden).

Samples (1g) of wet tissue or feed were soaked in 10 ml concentrated HNO3 (65% w/v, Suprapur, Merck, Darmstadt, Germany). Prior to analysis, complete digestion of the samples was performed using a microwave digestion system (CEM, Mars X-Press, NC, USA). The samples were heated in the microwave accelerated digestion system according to the following program: the power was ramped during 20 min from 100 W to 1200 W 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) prior to injection in the ICP-MS instrument. Standard solutions used for calibration curves were prepared from high purity standards (Multielement standard solution, Fluka Analytical, Sigma-Aldrich, St Louis, MO, USA).

The analytical procedure was validated using a Se recovery procedure (Georgiou and Kouparis, 1990) and two standard reference materials (NIST-RM 8414, bovine muscle powder and NIST-RM 1577c, bovine liver powder - LGC Standards; Promochem, Wesel, Germany). The Se recovery procedure was as follows: four 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 96-111% and the recoveries of the reference materials ranged from 93-120%, indicating the accuracy of the method.

3.3.3. Haematological analysis

Standard hematological analysis included determination of hematocrit, blood protein concentration and the leukocyte type (% of lymphocytes, heterophiles, monocytes, eosinophiles and basophiles). Hematocrit and blood protein concentration were determined using an ABX Pentra 400 bench top analyzer (Horiba-ABX, Montpellier, France). Leukocyte type (% of different white blood cells) was determined manually by light microscopy using a Neubauer chamber following a 1:20 dilution with the diluting solution (Turk's solution; 2% acetic acid v/v with a few drops of gentian violet) (WHO, 2000). The counting was performed by one haematologist who was blinded to the

blood probes examined. Lymphocytes, heterophiles, monocytes, eosinophiles and basophiles were counted and expressed as percentage of total white blood cells.

3.4. Statistical analysis

The statistical analysis was performed using SAS software (SAS Institute Inc., Cary NC, USA). All performance variates (body mass, feed intake, weekly body mass gain, feed conversion ratio, mortality) were analyzed by repeated measures ANOVA using treatment as the main factor and age of broilers as the repeated factor. Percentage data such as mortality were angularly transformed prior to analysis.

Selenium and Cd concentration data were log-transformed in order to achieve approximate normality. The vector of the dependent variables consisted of the concentrations of the trace elements while the fixed factors included treatment, age and tissue effects and their potential interactions.

Percentage haematological data were subjected to angular transformation prior to analysis. The vector of the response variables consisted of the haematological data while the fixed factors included treatment and age effects as well as their potential interactions. The basophil data appear to deserve special attention. In particular, the occurrence of non-zero measurements was rare and we analyzed these data via a Poisson log-linear model. In addition, the presence of a significant number of excess zeros suggested the use of a zero-inflated-Poisson (ZIP) model which is a mixture model. Specifically, the model assumes that the observation may be zero with probability π, or X with probability 1-π, where $X \sim Po(\theta)$ and Po denotes the Poisson distribution. The best model was chosen by comparing the deviance of the two models and by inspecting the 95% C.I. of π. The Poisson and ZIP models were fitted using the WinBUGS software (Lunn et al., 2000).

The elements' concentration data and the haematological ones are presented as the mean ± SEM of each of the main effects in turn, pooled for all the other main effects. Furthermore, in the results section all statistically significant interactions are presented. The statements of significance presented in this study were based on $P \leq 0.05$ unless otherwise stated.

3.5. Results

3.5.1. Performance of broilers

Addition of 10 ppm of Cd in the diet (T2) did not cause any significant difference in broiler's body mass compared to that of broilers fed diets without added Cd (T1) indicating that broilers supplemented with 0.3 ppm Se can tolerate low levels of Cd present in the diet. On the other hand, addition of 100 ppm of Cd (T3) significantly reduced ($P<0.001$) broilers' body mass compared to that of broiler's fed no added Cd (T1), by 11.95% at day 7 to 55.6% at day 42 (Table 6). The body mass of broiler's fed a diet with 100 ppm of Cd and 3 ppm of Se (T4) compared to that of broilers fed 100 ppm of Cd and 0.3 ppm of Se (T3) did not differ and was lower compared to that of broilers fed no or low levels of added Cd.

There were no statistically significant differences between the experimental treatments regarding the weekly mean feed consumption (WMFC) in the first week (0-7 days) of the experiment (Table 7). Thereafter, WMFC of broilers fed diets with high levels of added Cd (T3, T4) was significantly lower ($P<0.001$) compared to that of broilers fed no or low added Cd (T1, T2). Treatment T1 did not differ from T2 and similarly, T3 did not differ from T4.

The weekly mean body mass gain (WMBG) of broilers fed diets with high added Cd levels (T3 and T4) was significantly lower ($P<0.001$) compared to that of broilers fed no or low added Cd (T1 and T2) with no statistically significant difference between treatments T1 and T2 or T3 and T4 (Table 7). Overall, broilers fed no or low added Cd levels had better FCR values ($P<0.001$) compared to that of broilers fed high Cd level, irrespective of the level of Se present in the diet (Table 7). Mortality of broilers did not differ ($P>0.05$) between the four dietary treatments at any interval point or the whole period (Table 7).

3.5.2. Haematological Parameters

Overall, hematocrit and total blood protein of broilers (data pooled for age effects) did not differ between the four dietary treatments. Similarly, age of broilers did not affect the examined hematological parameters (Table 8). The significant treatment x age interaction ($P<0.001$) in

hematocrit values revealed that the hematocrit of broilers fed the T1 diet at 28 days was lower (28.30 % ± 0.81) compared to that at 42 days (33.50 % ± 1.77) and that the hematocrit of broilers fed the T4 diet at 28 days was higher (32.90 % ± 1.24) than that at 42 days (27.10 % ± 0.29). No significant differences were noted in leukocyte type indicating no treatment or age effect except for basophil values. In detail, basophil values (pooled data to exacerbate age effects) were 3.5 times lower ($P=0.051$) at 42 compared to 28 days. Similarly, lower basophil percentage ($P<0.05$) was noted in broilers fed the T4 diet compared to that of broilers fed the other experimental diets. The overall percentage representation of each white cell type was in the following order: lymphocytes > heterophiles > monocytes > eosinophiles > basophiles.

3.5.3. Selenium and Cd concentration in broiler's tissues

Broilers fed the diet with high Se concentration had increased tissue Se concentration (data pooled for age and tissue effects), by a factor of 5.4, compared to that in tissue broilers fed the low Se diets (Table 9). Age of broilers did not affect Se concentration. In detail, Se concentration of 6-week-old broilers (795 ± 117 ppb) did not differ from that in 4-week-old broilers (665 ± 83 ppb) (data pooled for treatment and tissue effects). Tissue type significantly affected Se concentration ($P<0.001$, data pooled for age and treatment to exacerbate tissue effects). Tissue Se concentration in descending order was kidney (1301 ± 217 ppb) > liver (934 ± 104 ppb)> breast muscle (393 ± 87 ppb)> blood (295 ± 38 ppb) and this pattern was noted for all treatments.

The concentration of Cd in 6-week-old broilers was 63% higher (19474 ± 3657 ppb) ($P<0.001$) compared to that in 4-week-old broilers (11928 ± 2375 ppb) (data pooled for treatment and tissue effects; Table 9). Tissue Cd concentration of broilers fed diets with 100 ppm of Cd was 14 times higher than that in broilers diets with 10 ppm Cd. Tissue type significantly affected Cd concentration ($P<0.001$, Table 9). Tissue Cd concentration in descending order was kidney (34625 ± 517 ppb)> liver (27769 ± 5149 ppb)> breast muscle (228 ± 36 ppb)> blood (183 ± 38 ppb) and this pattern was noted for all treatments.

3.6. Discussion

The occurrence of Cd is mainly associated with that of other metals since it is found in several, mainly Zn-rich, ores and is considered as an inescapable side-product of the metallurgy of these metals (Martelli et al., 2006). There are several national and international regulations controlling the risk of Cd exposure (EC, 1995; EC, 1998; EFSA, 2004) however, several reports indicate that in some cases, Cd levels in feed and manure can exceed the maximum permitted limits (Cang et al., 2004; Liu et al., 2005). Use of mineral premixes with high residues of Cd and application of animal manure, containing high Cd levels, as an organic fertilizer may be two routes of animal exposure (Sapunar-Postruznik et al., 2001; Cang et al., 2004; Liu et al., 2005). A study conducted in fourteen Chinese provinces revealed that the highest Cd concentrations measured in these areas were 27.60, 31.00 and 21.92 ppm in pig, dairy cattle and chicken feed, respectively (Li et al., 2010b).

Absorption and in turn tissue accumulation of dietary Cd is mainly influenced by dose, age, gender, species and nutritional status as well as by dietary intake of other elements that may interact with Cd (Klaassen et al., 1999; Groten et al., 1991; Andersen et al., 2004; Włostowski et al., 2005). The toxicity of a metal can be severely modulated by the interaction with other toxic or essential metals (WHO, 1996; Petersson-Grawe et al., 1997; Lopez-Alonso et al., 2004, 2007). This is the reason why Se has been studied as a remedy against Cd toxicity (Nolan and Brown, 2000; Jihen et al., 2008).

Selenium can be added to the broilers' diet either as an inorganic salt (sodium selenite or sodium selenate) or as an organo-Se compound more often in the form of Se-yeast (Zoidis et al., 2010). Organic Se is actively transported through intestinal membranes during absorption and excess of it can be accumulated in the tissues while excess of inorganic Se is excreted (Wolffram, 1999). Several studies showed that the organic form of Se has higher bioavailability and provides better antioxidant protection than inorganic form (Mahmoud and Edens, 2003; Lacour et al., 2004).

In the present study, addition to broilers' diets of 10 ppm Cd showed no negative effects on performance and mortality while addition of 100 ppm Cd caused significant negative effects on performance such as reduced body mass, decreased feed consumption and increased FCR. Although direct comparison with inorganic Se cannot be made since it was not used in the present study, previous work with inorganic Se indicated that 0.91 ppm of inorganic Se protected the hens against

a cumulative dose of 0.54 mg Cd per kg of body weight administered intraperitoneally but was ineffective against further Cd exposure (Nolan and Brown, 2000). The same authors reported that intraperitoneal Cd administration (0.32-1.62 mg/kg Cd) to hens did not cause negative effects on body weight, feed intake and hematocrit but reduced egg production. In another study by Li et al., (2010a), cocks were fed a diet with 10 ppm of inorganic Se or 150 ppm of Cd or a combination of those levels. The authors reported reduced final body weight in the Cd group compared to the other groups.

The reduced body weight reported in the present study is directly attributed to Cd toxicity. Several mechanisms have been reported for Cd toxicity. More specifically, Cd induces lipid peroxidation and interferes with some of the organism's enzymatic reactions, substituting Zn or other metals (Ognjanovic et al., 2010). Furthermore, it causes renal dysfunctions, hypertension, arteriosclerosis, damages in the nervous system, bone demineralization and endocrine dysfunctions (Lafuente et al., 2004; González-Weller et al., 2006; Schrauzer, 2009).

After oral exposure, Cd preferentially accumulates in the kidneys and liver (Lehman and Klaassen, 1986), a result which was corroborated in the present work. It is known that trace elements are bound to various ligands in cellular membranes, cytoplasm and blood and are transported and stored via several proteins including but not limited to albumin, ceruloplasmin, transferrin and metallothioneins (Southgate et al., 1989; Strachan, 2010). In the present study, the concentration of Cd deposited in the tissues was not affected by high Se level. This result is in line with studies with rats where inorganic Se did not affect deposited Cd level (Jihen et al., 2008; Ognjanovic et al., 2008). It is possible that 3 ppm of added Se could not ameliorate the toxic effects of 100 ppm added Cd.

This may be due to inadequate formation of certain Se-Cd complexes (Pappas et al., 2010). By examining solely the performance parameters of the current study, excess of Se fed to broilers did not ameliorate the negative effects of Cd. However, Se addition to broilers' feed did maintain mortality and haematological parameters to levels similar to that of broilers fed no added Cd and within the physiological range (Bounous and Stedman, 2000). It is known that broiler hematology has several distinctive characteristics such as red blood cells with nucleus and short life span and heterophil function parallels mammalian neutrophil function. In the present study, hematocrit ranged between 28 and 31%, values that are within the physiological values (22-35%).

The same applied for lymphocytes and heterophiles that accounted for the 80% of all white cells (Bounous and Stedman, 2000). Moreover, high Se addition in broilers' feed reduced basophil levels. Basopenia (a low basophil count) is generally difficult to be demonstrated since the normal basophil count is usually low. However, basopenia has been reported in association with stress-, and drug-induced reactions and autoimmune diseases (Grattan et al., 2003), but further research is indispensable to clarify the results of the present study. Other studies with concurrent Cd and inorganic Se addition in feed or drinking water showed that although Se could not counteract for Cd-induced decreased performance, it improved other examined parameters such as tissue structural damages, antioxidant defense and apoptosis (Jihen et al., 2008; Li et al., 2010a).

The present study indicated that Se can help against the negative effects of Cd but cannot counteract all of them indicating that more research is needed to establish the appropriate level of inclusion or use of Se in conjunction with other elements and antioxidants.

Table 5. Body mass (g) of the broiler population from 0 to 42 days, given per week

Age (days)	Body Mass (g)				P value
	T1	T2	T3	T4	
0	42 ± 0.67	41 ± 0.88	41 ± 0.73	41 ± 1.6	NS
7	159^{a} ± 2	154^{ab} ± 2	140^{b} ± 7	137^{c} ± 3	<0.001
14	399^{a} ± 5	389^{a} ± 6	267^{b} ± 8	272^{b} ± 11	<0.001
21	753^{a} ± 7	722^{a} ± 12	441^{b} ± 14	447^{b} ± 11	<0.001
28	1196^{a} ± 16	1174^{a} ± 22	626^{b} ± 20	663^{b} ± 23	<0.001
35	1643^{a} ± 35	1633^{a} ± 40	774^{b} ± 34	873^{b} ± 42	<0.001
42	2139^{a} ± 60	2178^{a} ± 80	950^{b} ± 47	1107^{b} ± 50	<0.001

Values are means ± SEM (n=5) of the main effects, pooled for all other main effects. Means within a row, within a main effect comparison, with different superscripts are different at P<0.05, unless otherwise stated.

T1= Treatment 1 with 0.3 ppm Se without added Cd.

T2= Treatment 1 with 0.3 ppm Se with10 ppm added Cd.

T3= Treatment 1 with 0.3 ppm Se with 100 ppm added Cd.

T4= Treatment 1 with 3 ppm Se with 100 ppm added Cd.

NS = non significant

Table 6. Broiler growth performance and mortality (%) determined for each week and for the whole experiment

	Age (days)						
	Treatment	**0-7**	**7-14**	**14-21**	**21-28**	**28-35**	**35-42**	**0-42**
WMFC[1] (g)	**T1**	182±2	350^{a} ± 3	566^{a} ± 2	752^{a} ±18	878^{a} ±37	1018^{a}±38	3747^{a} ± 89
	T2	193±2	346^{a} ± 6	551^{a} ±10	773^{a} ±18	896^{a} ±34	1074^{a}±54	3832^{a} ±115
	T3	192±4	247^{b} ± 9	352^{b} ±18	481^{b} ±28	472^{b} ±36	563^{b} ±20	2306^{b} ± 80
	T4	183±4	240^{b} ±14	376^{b} ±11	481^{b} ±23	566^{b} ±32	696^{b} ±34	2542^{b} ± 86
P value		NS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
WMBG[2] (g)	**T1**	117^{a} ±2	239^{a} ±3	355^{a} ±4	443^{a} ±14	447^{a} ±21	496^{a} ±29	2097^{a} ±60
	T2	113^{ab} ±2	235^{a} ±4	333^{a} ±7	451^{a} ±13	460^{a} ±21	545^{a} ±43	2137^{a} ±80
	T3	98^{bc} ±6	127^{b} ±8	174^{b} ±7	185^{b} ± 7	148^{b} ±24	176^{b} ±15	909^{b} ±47
	T4	96^{c} ±3	135^{b} ±8	175^{b} ±3	216^{b} ±15	210^{b} ±24	235^{b} ±17	1066^{b} ±50
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Mortality (%)	**T1**	0	0	0	1.3 ±1.3	0	0	1.3 ±1.3
	T2	0	0	0	1.3 ±1.3	0	0	1.3 ±1.3
	T3	4.0 ±4.0	0	1.3 ±1.3	2.6 ±1.6	0	0	8.0 ±3.2
	T4	1.3 ±1.3	0	1.3 ±1.3	0	0	0	2.6 ±1.6
P value		NS	NS	NS	NS	NS	NS	NS
FCR[3]	**T1**	1.56^{a} ±0.01	1.46^{a} ±0.1	1.60^{a} ±0.01	1.70^{a} ±0.02	1.97^{a} ±0.03	2.06^{a} ±0.06	1.78^{a} ±0.01
	T2	1.71^{ab} ±0.02	1.47^{a} ±0.1	1.66^{a} ±0.01	1.71^{a} ±0.01	1.95^{a} ±0.01	1.99^{a} ±0.05	1.79^{a} ±0.01
	T3	1.98^{bc} ±0.08	1.97^{b} ±0.13	2.02^{b} ±0.05	2.60^{b} ±0.12	3.39^{b} ±0.40	3.26^{b} ±0.23	2.54^{b} ±0.06
	T4	1.92^{c} ±0.07	1.78^{b} ±0.01	2.15^{b} ±0.07	2.25^{b} ±0.10	2.80^{ab} ±0.28	3.01^{b} ±0.20	2.39^{b} ±0.06
P value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means ± SEM (n=5) of the main effects, pooled for all other main effects. Means within a column, within a main effect comparison, with different superscripts are different at $P<0.05$, unless otherwise stated. Percentage data are untransformed means ± SEM. Statistical differences indicated in the table and P values refer to transformed data.

NS = non significant

[1]WMFC = weekly mean feed consumption (g)

[2]WMBG = weekly mean body mass gain (g)

[3]FCR = feed conversion ratio (feed intake: body mass gain)

Table 7. Hematocrit (%), blood protein concentration (g dl^{-1}), and leukocyte type (% of total white blood cells) for the different main effects

Factor studied	Hematocrit (%)	Total protein (g dl^{-1})	Heterophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)
Age (days)							
28	29.83±0.64	9.29±0.26	43.10±2.01	49.42±2.04	5.68±0.44	1.45±0.18	0.35^{a} ±0.10
42	29.13±0.79	9.40±0.27	43.30±1.96	50.57±2.01	4.90±0.47	1.13±0.15	0.10^{b} ±0.06
Treatment							
T1	30.90±1.26	9.97±0.49	43.70±2.60	49.10±3.29	5.75±0.67	1.00±0.22	0.45^{a} ±0.17
T2	28.60±0.60	9.03±0.14	42.60±3.05	51.35±3.05	4.65±0.48	1.20±0.13	0.20^{a} ±0.08
T3	28.40±0.86	9.31±0.38	43.50±3.29	48.40±2.88	6.20±0.63	1.65±0.32	0.25^{a} ±0.11
T4	30.00±1.13	9.09±0.36	43.00±2.52	51.15±2.35	4.55±0.70	1.30±0.23	0.00^{b} ±0.00
Source of Variation	**P Value**						
Treatment	NS	NS	NS	NS	NS	NS	P<0.05
Age	NS	NS	NS	NS	NS	NS	P=0.051
Treatment X Age	P<0.001	NS	NS	NS	NS	NS	NS

Values are means ± SEM (n=5) of the main effects, pooled for all other main effects. Means within a column, within a main effect comparison, with different superscripts are different at P<0.05, unless otherwise stated. Percentage data are untransformed means ± SEM. Statistical differences indicated in the table and P values refer to transformed data.

NS = non significant

Table 8. Selenium and Cd concentration (ppb) in blood, liver, kidney and breast tissue at 4^{th} and 6^{th} weeks of age

Factor Studied	**Trace Element**	
	Se	**Cd**
Age (weeks)		
4^{th}	665 ± 83	11928^{a} ± 2375
6^{th}	795 ± 117	19474^{b} ± 3657
Treatment		
T1	388^{a} ± 38	26^{a} ± 4
T2	373^{a} ± 42	2114^{b} ± 474
T3	281^{b} ± 33	31270^{c} ± 5427
T4	1880^{c} ± 186	29393^{c} ± 5172
Tissue		
Blood	295^{a} ± 38	183^{a} ± 38
Liver	934^{b} ± 104	27769^{b} ± 5149
Kidney	1301^{c} ± 217	34625^{c} ± 517
Breast	393^{d} ± 87	228^{a} ± 36
Source of Variation	**P Value**	
Age	P=0.403	P<0.001
Treatment	P<0.001	P<0.001
Tissue	P<0.001	P<0.001
Treatment X Age	P<0.001	P=0.689
Treatment X Tissue	P<0.001	P<0.001
Age X Tissue	P<0.001	P<0.001
Treatment X Age X Tissue	P<0.001	P=0.001

Data were transformed (log transformation) prior to analysis. Values are the non transformed means ± SEM (n=5) of the main effects, pooled for all other main effects. Means within a column, within a main effect comparison, with different superscripts are different at P<0.05, unless otherwise stated. Statistical differences indicated in the table and P values refer to transformed data.

PICTURES

Picture (1): Body size differences between broilers orally administered or not with Cd

Picture (2): Body size differences between broilers orally administered or not with Cd

Picture (3): Chickens' units

Picture (4): Chickens' farm design

Picture (5): Chickens' units

Picture (6): Chickens at first day

Picture (7): Chickens at first day

Part C

Experiment 2

4. The effects of Zn, Se and Cd feed supplementation on broilers' performance, hematological parameters and accumulation of essential and toxic elements in various tissues

4.1. Abstract

This study was designed to assess: i) if organic selenium (Se) and Zn can protect against the toxic effects of cadmium (Cd) and ii) to examine if Se, Zn and Cd addition to chickens' diet affect the accumulation of Se Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn in the tissues.

A total of 180 day-old, as hatched, broilers were randomly distributed in three dietary treatments with 4 replicate pens per treatment. In T1 treatment, chickens were fed a diet with 0.3 ppm added Se (as Se yeast), 100 ppm Zn (as ZnO), without added Cd. In T2, chickens were fed a diet with 0.3 ppm Se, 150 ppm Zn and 50 ppm Cd (as $CdCl_2$). In T3, chickens were fed a diet with 0.5 ppm Se, 150 ppm Zn and 50 ppm Cd. On the 4^{th} and 6^{th} week, two broilers per replicate pen were sacrificed for whole blood, liver, kidney and breast samples. Body mass, feed conversion ratio and mortality were assessed and hematological analyses were performed. Se, Zn and Cd levels, as well as the levels of fourteen additional essential and toxic elements in tissues were analyzed by inductively coupled plasma mass spectrometry.

Addition of 50 ppm Cd to the diet led to an impairment of broilers' performance which could not be ameliorated by Se and Zn. Nonetheless, the examined hematological parameters ranged within physiological values revealing no negative health effects after simultaneous Cd, Se and Zn addition.

The present study indicated that Se and Zn can partially help against the negative effects of Cd, at least at the levels of Cd tissue accumulation, but cannot counteract all of its negative effects. Furthermore, the study revealed several correlations between essential, probably essential and toxic metals illustrating the importance of the balance between pro-oxidants and antioxidants.

4.2. Introduction

Hudson et al. (2005) reported that reduced weight gain, shortening and thickening of leg bones, reduced bone ash and poor feather development may occur when Zn intake by chicks is insufficient. Furthermore, providing a diet with adequate Zn to broilers may negate adverse effects of low Zn intake

by breeder hens (Hudson et al., 2005). Suttle (2010) showed that zinc is required for the structural and functional integrity of over 2000 transcription factors. Rojas et al. (1995) indicated that lambs had higher Zn concentrations in different tissues than the control group when lambs supplemented with Zn-lysine (360 mg Zn/ kg DM), when basal diet contained a range from 16 to 20 mg Zn/ kg DM. Furthermore, feed Zn supplementation with 15 mg Zn/kg DM was required for obtaining higher immune response in lambs fed a basal diet containing 29.28 mg Zn/kg DM as Nagalakshmi et al. (2009) indicated. In addition, Chesters (1997) indicated that Zn could affect animal growth, immune system, and reproduction by influencing gene expression of proteins and enzyme activity or by its influence on signal transduction of mitogenic hormones, gene transcription and RNA synthesis as also Macdonald (2000) reported.

The NRC (1994) estimates the Zn requirement for broiler chickens as 40 mg kg^{-1} of diet. Conversely, Burrell et al. (2004) reported improved performance when broilers consumed diets formulated to contain 110 mg Zn kg^{-1}. For dairy animals, the NRC (1985) recommended a level of 33 mg Zn/kg DM in the diet for growing lambs as well as for calves (NCR, 2001). Accordingly, Spears and Kegley (2002) indicated that feed supplementation with 25 mg Zn/kg DM significantly improved growth rate of calves. In addition Lardy et al. (1992) and Coa et al. (2000) showed that Zn supplementation obtained through organic sources caused higher retention and tissue concentrstion compared with inorganic sources ($ZnSO_4$ or ZnO). Furthermore, Hempe and Lousins (1989) indicated that increasing tissue supply of Zn would improve animal productivity by transferring Zn methionine completely from the intestinal lumen into mucosal cells.

Cadmium has been shown to interfere with a variety of biological processes. Chronic oral exposure in the adult animal produces kidney damage (Axelsson et al., 1966), hypertension (Schroeder, 1965), anaemia (Wilson et al., 1941), tumors (Schroeder et al., 1964) and in conjunction with certain dietary deficiencies, osteomalacia (Nogawawa et al., 1975). It affects growing animals and the fetuses of pregnant animals by producing anaemia and growth retardation (Webster, 1978), while chronic respiratory exposure may cause emphysema (Friberg, 1950).

Many of these toxic effects of cadmium can be prevented by prior or simultaneous exposure to other chemicals, in particular, metals (Nordberg et al., 1978). Zinc has been shown in laboratory animals to protect against some of the chronic effects of cadmium such as kidney damage (Vigliani, 1969) and in the growing animal to partially prevent the anaemia and growth retardation (Bunn and Matrone, 1966). It also protects against some acute effects of cadmium such as testicular necrosis (Parizek, J. 1957), foetal malformations (Ferm and Carpenter, 1968) and placental destruction (Chiquoine, 1965).

Cadmium is absorbed in the small intestine by the same mechanisms as are essential elements, such as Ca, Fe, Cu and Zn (Andersen et al., 1994; Fairweather-Tait, 1995; Brzoska and Moniuszko-Jakoniuk, 1998). Cd–Zn interactions at the stage of their absorption from the gastrointestinal tract are closely related to the affinity of both metals for intestinal MT, the ability to synthesize this protein and competition for uptake (Min et al., 1991; Ohta and Cherian, 1991; Andersen et al., 1994; Elsenhans et al., 1994). In consequence, Cd disturbs Zn absorption. In experimental studies it has been demonstrated that even low-level Cd intake can inhibit Zn absorption (Coppen-Jaeger and Wilhelm, 1989). Low dietary Zn is an important factor increasing Cd absorption and retention (Fox et al., 1984; Panemangalore, 1993). Fox et al. (1984) have shown that low dietary Zn (11 mg/kg) led to increased ^{109}Cd retention in duodenum, jejunum–ileum and liver of young Japanese quails.

The dosing of rats with 5 mg Cd/l of drinking water (or less) is within the range of human environmental exposures, which are up to 30 mg/day in uncontaminated areas and up to 200 mg/day in Cd-contaminated areas (WHO, 1992; Brzoska et al., 2000). On the basis of the available data it can be concluded that protective action of Zn supplementation against Cd accumulation in conditions of oral exposure is due, at least in part, to immobilisation of Cd by Zn induced intestinal MT (Ohta and Cherian, 1991).

Therefore, the aim of this study was to examine the effects of the maximal allowed levels of organic Se and Zn supplemented in feed on performance and health of broilers as well as the accumulation of 17 elements and their correlations in various broiler tissues after orally induced Cd toxicity.

4.3. Materials and Methods

4.3.1. Animals

One hundred eighty 1 day-old as hatched Cobb 500 chickens, obtained from a commercial hatchery, were used in a similar experiment to determine the effects of cadmium, zinc, and selenium supplementation in feed on productive and biochemical parameters. There were three treatments namely T1, T2 and T3 randomly allocated in the house. Each treatment had four pens, (2m length X 1m width), with twelve pens in total. There were fifteen chickens per pen, sixty chickens per treatment.

4.3.2. Diets

All aspects of chickens' feed were the same for the three treatments, except for the levels of the three supplemented elements. In T1 treatment, the chickens were fed a diet containing 0.3 ppm of organic Se and 100 ppm Zn, without any Cd. In T2, the chickens were fed a diet with 0.3 ppm of organic Se, 150 ppm Zn, as well as 50 ppm of Cd. In T3, chickens were fed a diet with 0.5 ppm of organic Se, 150 ppm of Zn and 50 ppm of Cd. Moreover, Se was added as a Se-yeast (SelPlex®, Alltech Inc., Nicholasville, KY, USA), Zn was added as a ZnO while Cd was added as a $CdCl_2$ (Sigma-Aldrich, St Louis, MO, USA) (Table 9).

4.3.3. Design

The duration of the experiment was six weeks with housing and care of the chickens, conforming to the guidelines of the bioethics committee Faculty of Animal Science and Aquaculture of the Agricultural University of Athens. According to Cobb's management manual the chickens were raised in a house where the light and ventilation were controlled. The chickens were fed a starter-, grower- and finisher diet until the 10^{th}, 20^{th} and the 42^{nd} day of their lives, respectively (Table 9).

Feed and water were provided *ad libitum*. The lighting program was twenty three hours of light and one hour of dark. Stocking density was according to EU legislation. At the end of the 42^{nd} day, three chickens per pen (12 per treatment) were individual weighed, and sacrificed. Liver and kidneys were weighed to calculate the organ to body weight ratio. Moreover, liver, breast, kidney and blood samples were collected for trace element analysis.

At weekly intervals, birds were weighted, their body mass recorded and weekly mean body mass gain (WMBG) calculated. Moreover, feed intake was measured weekly and weekly mean feed consumption (WMFC), and feed to gain ratio (FCR) were calculated too.

At the end of the experiment in day 42^{nd} three birds per pen were sacrificed so the samples were collected. Liver, kidney, blood and breast were collected to investigate the amount of Se, Zn and Cd determination. Birds were inspected daily and the mortality was recorded daily on the appropriate data capture form. Total mortality was calculated as the number of birds that died throughout the study compared to the initial number of birds placed corrected for birds removed for blood collection

4.3.4. Hematological analysis

Standard hematological analysis included determination of hematocrit, blood protein concentration and the leukocyte type (% of lymphocytes heterophiles, monocytes, eosinophiles and basophiles). Hematocrit and blood protein concentration were determined using an ABX Pentra 400 bench top analyzer (Horiba-ABX, Montpellier, France). Leukocyte type (% of different white blood cells) was determined manually by light microscopy using a Neubauer chamber following a 1:20 dilution with the diluting solution (Turk's solution; 2% acetic acid v/v with a few drops of gentian violet) (WHO, 2000). The counting was performed by one hematologist who was blinded to the blood probes examined. Lymphocytes, heterophiles, monocytes, eosinophiles and basophiles were counted and expressed as percentage of total white blood cells.

4.4. Determination of elements

The samples were heated in the microwave accelerated digestion system according to the following program: the power was ramped during 20 min from 100 W to 1200 W 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) prior to injection in the ICP-MS instrument. Standard solutions used for calibration curves were prepared from high purity standards (Multielement standard solution, Fluka Analytical, Sigma-Aldrich, St Louis, MO, USA).

Selenium, Cd, Sb, As, Ca, Cr, Co, Cu, Fe, Pb, Li, Mg, Mn, Mo, Ni, V and Zn were determined in samples using inductively coupled plasma mass spectrometry, ICP-MS (Perkin Elmer, Elan 9000, Perkin Elmer Life and Analytical Sciences Inc, Waltham, MA, USA) as described previously. The instrumental parameters of the equipment used were: nebuliser flow 0.775 l min^{-1}, vacuum pressure 1.5×10^{-5} Torr, lens voltage 950W, 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 prior to feeding and milled prior to analysis through a 1 mm sieve (Cyclotec, 1093 sample mill, Tecator, Höganäs, Sweden). Samples (1g) of wet tissue or feed were soaked in 10 ml concentrated HNO3 (65% w/v, Suprapur, Merck, Darmstadt, Germany). Prior to analysis, complete

digestion of the samples was performed using a microwave digestion system (CEM, Mars X-Press, NC, USA).

The analytical procedure was validated using a Se recovery procedure (Georgiou and Kouparis, 1990) and two standard reference materials (NIST-RM 8414, bovine muscle powder and NIST-RM 1577c, bovine liver powder - LGC Standards; Promochem, Wesel, Germany). The Se recovery procedure was as follows: four samples from the same liver tissue (1 g each) were spiked with 250, 500 and 750 µl of Se standard solution of 4 ppm, 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 96-111%.

4.5. Statistical Analysis

The Statistical Analysis System- SAS (2012) was used to determine the effects of different treatments on the study parameters. The data were analyzed using a multivariate linear model where Se, Cd, Ca, Co, Cu, Fe, Li, Mg, Mn, Sb, As, Cr, Pb, Mo, Ni, V and Zn concentrations represented the response variables, while treatment and tissue were the fixed factors. All performance variates (body mass, feed intake, weekly body mass gain, feed conversion ratio, mortality) were analyzed by repeated measures ANOVA using treatment as the main factor and age of broilers as the repeated factor. Percentage data such as mortality were angularly transformed prior to analysis.

Completely randomized design (CRD) was used for the data analysis. Duncan's multiple range (1955) test was used to significant compare between means in this study.

Statistical model:

$$Yij = \mu + Ti + eij$$

Where Y denotes Se, Cd, Ca, Co, Cu, Fe, Li, Mg, Mn, Sb, As, Cr, Pb, Mo, Ni, V and Zn concentrations.

The elements' concentration data and the hematological ones are presented as the mean ± SEM of each of the main effects in turn, pooled for all the other main effects. The statements of significance presented in this study were based on $P \leq 0.05$ unless otherwise stated. Furthermore, in the results section all statistically significant interactions are presented. Organ weights were also tested statistically using body weight as a covariant to evaluate the effect of body weight on organ weight.

4.6. Results

Presented are in Table 9 the composition (g kg^{-1}), the calculated analysis and the determined Se, Zn and Cd concentration (ppb) of the experimental broiler diets

Table 10 depicts the concentrations of 17 elements measured in experimental diets including those added to the broiler feed. The concentration of Se in T1 was significantly higher in comparison to treatments T2 and T3. Furthermore, Cd concentration in T1 was significantly lower when compared to the other treatments. On the other hand, there were no significant differences in the Zn concentrations between all treatments. Moreover, there were many elements which varied greatly in concentrations between the treatments, as shown in Table 10.

The levels of selected elements within different tissues (liver, kidney, breast and blood) determined are shown in tables 11, 12, 13 and 14, respectively. The results displayed a wide range of metal concentrations, all depended on the different levels of dietary Se, Zn, and Cd according to the treatments of the experiment. In comparison to T2 and T3, T1 showed the highest levels of Se within the liver tissues. This result presents the idea that the Se added to the feed was spent to bind with high levels of Cd in order to protect the body from the toxic effects of Cd in T1 and T2 (Table 14). In addition, the levels of Ca were notably high in all three treatments. On the other hand, concentrations of Fe were higher in T2 and T3 in relation to T1, which may be related to the high doses of Cd given to both treatments. This information could provide a picture of the normal levels of Fe in the feed. There had been no other significant effects on the other elements recorded within the liver tissues, Co, Cu, Li, Mg, Mn, Sb, As, Cr, Pb, Mo, Ni, V (Table 14).

When examining the kidney tissues it was noted that the levels of Se were increased in T1 compared to T2 and T3, presenting the idea that Se could help the body against toxicity of Cd. On the other hand, Ca, Co, Fe, Li, Mg, Mn, As, Cr, Pb, Mo and V levels were decreased in T2 and T3 compared to T1. The high levels of Cd used in this study might have affected the concentrations of those

elements either by binding with these elements inside the tissue or by decreasing the chickens' appetite throughout the experiment. Resulted were decreased amounts of these elements in kidney (Table 13). Moreover, the Cu level in this tissue showed different results than other elements. It increased in T2, but decreased in T1 and T3. Similar to this result, the Sb level was high in T3 when compared with T1 and T2 (Table 13).

The third tissue examined in this study was the breast tissue. It was shown that the Se and Cd levels were similar to the levels in liver and kidney. On the contrary, the Cd levels in T3 were lower compared with T2. Both treatments (T2 and T3) had 50 ppm Cd added to the feed, but the Cd in T3 was affected by the high levels of Se and Zn in T3. Fe and Mn showed the largest increase in T3 because of the positive relationship with the high levels of Se, Zn and Cd in this treatment. Ni concentration however, ranged different than the other elements, as it was higher in T1 and T3 than in T2, Otherwise, the rest of the elements in this tissue didn't show any significant changes in relation to Se, Zn and Cd added to the feed. These elements were Ca, Co, Cu, Li, Mg, Sb, As, Cr, Pb, Mo V and Zn.

Finally, the concentrations of the selected elements in blood were examined. Many elements like Se, Ca, Co, Cu, Mn, Cr, Ni and Zn had higher concentrations in T1 compared to T2 and T3. The concentrations of Ni, Zn, Co, Cu and Mn showed the highest levels in T1. On the other hand, Cd and Mo had increased levels in both T2 and T3, but not in T1. The concentrations of the rest of the elements examined didn't show any significant changes in response to Se, Zn and Cd supplementation in blood such as Fe, Li, Mg, Sb, As, Pb and V.

Table 9. Composition (g kg^{-1}), calculated analysis and determined Se, Zn and Cd concentration (ppb) of the experimental broiler diets

Ingredients (g kg^{-1})	**Starter (0-10 d)**	**Grower (11-22 d)**	**Finisher (23-42 d)**
Wheat	150.0	500.0	550.0
Corn	460.0	157.4	146.3
Soybean meal	313.4	274.6	224.5
Fish meal	12.5	-	-
Soybean oil	24.4	28.7	21.7
Vegetable oil	-	6.2	25.0
Bicalcium phosphate	14.4	9.7	9.7
Limestone	12.9	11.9	12.2
Lysine	3.1	2.8	2.4
Methionine	3.3	2.7	2.2
NaCl	2.0	2.0	2.0
Vitamin + mineral premix[1]	4.0	4.0	4.0
Calculated Analysis (g kg^{-1} DM**)**			
Dry matter (g kg^{-1})	874.0	876.0	875.5
ME (MJ kg^{-1})	12.9	13.2	13.6
CP (g kg^{-1})	217.0	202.0	185.2
Sodium (g kg^{-1})	1.6	1.5	1.5
Ca (g kg^{-1})	10.0	8.5	8.5
Available P (g kg^{-1})	5.0	4.2	4.2
Methionine	6.7	5.7	5.0
Methionine+cysteine (g kg^{-1})	10.3	9.3	8.4
Lysine (g kg^{-1})	13.8	12.1	10.7

Treatment	**Se added[2] (ppb)**	**Zn added[3] (ppb)**	**Cd added[4] (ppb)**
T1	300	100,000	0
T2	300	150,000	50,000
T3	500	150,000	50,000

Treatment	**Se determined[5] (ppb)**	**Zn determineded[5] (ppb)**	**Cd determined[5] (ppb)**
T1	359	94,000	1007
T2	439	151,000	43,570
T3	622	141,000	35,670

[1]Premix supplied per kg of diet: 12,000 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, 0.025 mg 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.

[2]Se was added in the form of Sel-Plex® (Alltech Inc, Nicholasville, KY, USA)

[3]Zn was added in the form of ZnO_2

[4]Cd was added in the form of $CdCl_2$

[5]The determined Se, Zn and Cd contents represent pooled data from all three diets (starter, grower and finisher)

Table 10: The concentration (ppb) of 17 elements determined in T1, T2, and T3 experimental diets

Parameters	Mean ± SE			P-value
	T1	T2	T3	
Se	586±49 [a]	459±37 [b]	438±32 [b]	0.0194
Cd	48±4.6 [b]	24074±3737 [a]	21056±3356 [a]	<.0001
Ca	46607±3440	39004±2665	39007±1844	0.0788
Co	23±3.5	19±2.1	18±1.8	0.3909
Cu	1860±356.7	1962±248.8	1771±228.0	0.8927
Fe	128214±14918.5 [a]	87749±12215.7 [b]	91030±12889.3 [ab]	0.0631
Li	3.3±0.06 [a]	2.9±0.07 [b]	2.7±0.07 [c]	<.0001
Mg	186427±13373	175186±13545	162276±14703	0.4708
Mn	1553±211.3	1165±164.5	1118.7±159.7	0.1764
Sb	1.0±0.009 [b]	0.95±0.01 [b]	1.08±0.04 [a]	0.0005
As	9.26±0.23 [a]	8.86±0.25 [a]	8.2±0.21 [b]	0.0059
Cr	30.6±1.6 [a]	26.4±1.6 [a]	18.9±1.74 [b]	<.0001
Pb	17.9±0.16 [a]	17.3±0.27 [a]	15.5±0.51 [b]	<.0001
Mo	342.8±36.8[a]	477.1±171	289.8±31.84	0.4156
Ni	15.5±3.64[a]	8.58±1.5 [b]	7.7±0.77 [b]	0.0357
V	3.1±0.06	3.1±0.07	3.01±0.07	0.4125
Zn	16717±1911.9	19339±2742.5[a]	13799±2046.06	0.2267
Means within a row with different superscripts are different at P<0.05				

Table 11: The concentration (ppb) of 17 elements determined in broilers' blood

Parameters	Mean ± SE			P-value
	T1	T2	T3	
Se	307.8±26.7 [a]	243.08±9.8 [b]	277.03±11.8 [ab]	0.0834
Cd	29.4±2.3 [c]	114.2±4.2 [a]	100.3 ±2.2 [b]	<.0001
Ca	58577±3369.09 [a]	48318±2965.8 [b]	37937±263.4 [c]	0.0011
Co	16.3±10.8	6.5±0.29	7.0±0.5	0.4881
Cu	1611±1310.6	319±35.1	245±6.03	0.3957
Fe	237140±11771	223650±5989	218026±3265	0.2613
Li	2.9±0.1	2.8±0.02	2.7±0.02[a]	0.1461
Mg	37820±1254.1	37019±1110.4	36764±644.02	0.7599
Mn	92.4±15.1 [a]	66.03±3.6 [ab]	60.1±1.5 [b]	0.0714
Sb	1.02±0.02	1.0±0.000	1.0±0.000	0.4053
As	11.0±0.1 [a]	10.7±0.1 [a]	9.2±0.39 [b]	0.0019
Cr	17.7±0.5 [a]	16.1±0.5 [a]	12.7±0.6 [b]	0.0004
Pb	18.5±0.4	18.6±0.7	18.0±0.04	0.6001
Mo	134.5±12.02	154.2±16.09	147.2±6.02	0.5303
Ni	433.2 ±425.1	10.4 ±6.3	4.9±1.04	0.4047
V	3.6750±0.094 [a]	3.6750±0.047 [a]	3.3250±0.085 [b]	0.0172
Zn	4635±1982.35	2419±383.325	1898±207.306	0.2663

Means within a row with different superscripts are different at P<0.05T1= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T2= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T3= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0.

Table12: The concentration (ppb) of 17 elements determined in broilers' breast

Parameters	Mean ± SE			P-value
	T1	T2	T3	
Se	217.05±8.04	207.4±8.5	239.6±13.2	0.1273
Cd	21.6±0.2 [b]	331.8±106.1 [a]	240.03±17.1 [a]	0.0173
Ca	25534±1950	21233±6660	22924±2609	0.7794
Co	3.4±0.1	4.1±0.1	6.7±2.3	0.2446
Cu	335.6±9.6	317.1±19.1	384.7±84.4	0.6329
Fe	12731±308.2	11081±351.3	35277±23961.2	0.4208
Li	3.2±0.2	2.7±0.07	2.8±0.1	0.1172
Mg	282703±5252	282746±4286	296240±17338	0.6075
Mn	174.3±.4.1	151.5±5.9	238.1±59.1	0.2364
Sb	1.0±0.000	0.9±0.04	0.97±0.08	0.4377
As	7.8±0.07 [a]	6.6±0.29 [b]	6.7±0.4 [b]	0.0410
Cr	30.7±1.9	30.9±0.7	34.0±2.7	0.4578
Pb	17.6±0.1 [a]	15.9±0.4 [b]	16.05±0.6 [b]	0.0644
Mo	63.5±19.08	60.2±11.5	56.05±3.7	0.9220
Ni	17.2±4.9 [a]	6.1±0.39 [b]	12.2±2.1 [ab]	0.0931
V	2.75±0.02 [a]	2.42±0.09 [b]	2.45±0.1 [b]	0.0389
Zn	4519.7±116.5	4403.1±805.5	4744.1±340.2	0.8920

Means within a row with different superscripts are different at P<0.05T1= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T2= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T3= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0.

Table13: The concentration (ppb) of 17 elements determined in broilers' kidney

Parameters	Mean ± SE			P-value
	T1	T2	T3	
Se	923.13±32.494 [a]	595.03±21.680 [b]	468.03±14.713 [c]	<.0001
Cd	97±7.121 [b]	57955±1785.52 [a]	51375±4742.62 [a]	<.0001
Ca	73369±6965.30 [a]	52303±4452.91 [b]	45289±3546.84 [b]	0.0104
Co	44.900±0.801 [a]	36.500±3.502 [b]	27.050±1.560 [c]	0.0012
Cu	2357.5±49.545 [b]	3425.0±222.595 [a]	2733.5±177.116 [b]	0.0044
Fe	54158±4780.54 [a]	36201±1392.96 [b]	25278±4574.23 [b]	0.0017
Li	3.5250±0.197 [a]	2.6750±0.062 [b]	2.1000±0.234 [b]	0.0012
Mg	209803±3707.91 [a]	157996±3675.25 [b]	108244±2791.41 [c]	<.0001
Mn	2707.7±68.417 [a]	1802.4±135.685 [b]	1516.4±102.011 [b]	<.0001
Sb	0.9750±0.025 [b]	0.9250±0.025 [b]	1.4000±0.135 [a]	0.0045
As	10.2000±0.470	9.4500±0.193	8.4000±0.803	0.1197
Cr	32.600±1.289 [a]	19.475±1.698 [b]	4.475±0.654 [c]	<.0001
Pb	17.900±0.353 [a]	17.075±0.347 [a]	11.375±2.137 [b]	0.0106
Mo	625.65±29.059 [a]	513.48±20.362 [b]	403.30±10.425 [c]	0.0002
Ni	10.3000±0.930 [a]	6.9500±0.221 [b]	5.6750±0.534 [b]	0.0016
V	3.3000±0.081	3.2250±0.047	3.0750±0.262	0.6194
Zn	24578.1±680.544 [a]	23089.8±380.550 [a]	12151.2±491.070 [b]	<.0001

Means within a row with different superscripts are different at P<0.05

T1= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T2= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T3= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0.

Table14: The concentration (ppb) of 17 elements determined in broilers' liver

Parameters	Mean ± SE			P-value
	T1	T2	T3	
Se	897.88±24.8 [a]	791.88±22.9 [b]	769.48±26.4 [b]	0.0115
Cd	43±2.2 [b]	37896±2508 [a]	32510±3213 [a]	<.0001
Ca	28950±4871 [b]	34162±2634 [b]	49879±3307 [a]	0.0083
Co	26.10±0.4 [b]	28.9±0.7 [a]	30.6±1.2 [a]	0.0154
Cu	3136.4±197	3788.6±106	3723.3±335	0.1490
Fe	208825±20857[a]	80063±4747 [b]	85538±6562 [b]	<.0001
Li	3.4750±0.17	3.4250±0.17	3.1000±0.09	0.2152
Mg	215381±4564	222984±6864	207859±6711	0.2702
Mn	3238.3± 83.5[a]	2640.5±158.8 [b]	2660.3±242.9 [b]	0.0639
Sb	1.00±0.0[a]	0.97±0.02 [ab]	0.92±0.02 [b]	0.0751
As	7.95±0.13	8.62±0.34	8.45±0.31	0.2616
Cr	41.2±1.27 [a]	39.2±3.48 [a]	24.4±0.73 [b]	0.0008
Pb	17.75±0.23 [a]	17.45±0.25 [ab]	16.62±0.35 [b]	0.0522
Mo	547.5±22.8	1180.3±649.7	552.8±65.8	0.4295
Ni	24.98±13.21	10.93±0.86	8.18±0.45	0.2984
V	2.82±0.06 [b]	3.12±0.11 [ab]	3.22±0.13 [a]	0.0675
Zn	33136±828 [b]	47445±2566 [a]	36402±2581 [b]	0.0028

Means within a row with different superscripts are different at P<0.05

T1= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T2= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0. T3= Se with 0.3, ppm, Zn with 100 ppm and Cd with 0.

The correlation between elements was studied in this project. Se was positively correlated with Cd, Ca, Cu, Li and Zn. On the other hand, Se didn't show any correlation with Fe, Sb and V. Moreover, Zn had positive correlation with Se, Cd, Cu and Li. Also, Cd was positively correlated with Cu, Sb, and Zn but negatively with Fe and Li. In addition, Cd didn't have any correlation with Ca and V. Furthermore, Ca had a positive correlation with Se, Cu, Fe, Li, and V but not with Cd, Sb and Zn. On the other hand, Cu had negative correlation with Fe but positive with Li and Zn. Additionally, there weren't any correlations between Cu with Li, Sb and V in this study.

Fe had strong correlation with V but no correlation with Li, Sb and Zn. On the other hand, Li showed strong correlation with Sb (negative), V and Zn (positive). Both Sb and V didn't show any correlation with V and Zn, respectively (Table 15).

Table 15: Correlations between the concentrations of Se, Zn and Cd with six other elements

	Cd	**Ca**	**Cu**	**Fe**	**Li**	**Sb**	**V**	**Zn**
Se	0,341**	0,343**	0,850**	0,009	0,444**	-0,022	0,128	0,877**
Cd		0,157	0,724**	-0,362**	-0,257**	0,316**	0,077	0,489**
Ca			0,213*	0,174*	0,190*	0,034	0,572**	0,126
Cu				-0,166*	0,178*	0,126	0,059	0,897**
Fe					0,099	0,047	0,510**	-0,108
Li						-0,275**	0,186*	0,391**
Sb							0,083	-0,097
V								0,029
Zn								

**. Correlation is significant at P< 0.01 (2-tailed).

*. Correlation is significant at P< 0.05 (2-tailed).

No significant differences were noted in the examined hematological characteristics leukocyte type and hematocrit indicating no treatment effects except for eosinophil % values which were about 3-fold decreased (P<0.05) in T3 compared to T1 (Table 16). The overall percentage representation of each white cell type was in the following order: herophils > lymphocytes > monocytes >eosinophiles > basophiles.

Table16. The effect of different treatments on blood parameters of broiler chickens (ppm)

Parameters ppm	**Mean ± SE**			**P-value**
	T1	**T2**	**T3**	
Hematocrit **(%)**	34.0 ± 1.7	35.75 ± 0.8	32.00 ± 0.4	0.128
Heterophil **(%)**	36.75 ± 2.8	46.50 ± 3.9	48.00 ± 5.5	0.183
Lymphocyte **(%)**	40.00 ± 2.8	39.00 ± 5.6	39.50 ± 4.8	0.988
Monocyte**(%)**	14.00 ± 3.1	8.25 ± 1.3	9.75 ± 1.3	0.190
Eosinophil **(%)**	7.75 ± 0.63 [a]	5.25 ± 1.65 [ab]	2.50 ± 0.29 [b]	0.018
Basophil **(%)**	1.50 ± 0.5	1.00 ± 0.4	0.25 ± 0.2	0.138

Means within a row with different superscripts are different at P<0.05

Table 17 displays the effect of different levels of Se, Zn and Cd on body weight gain (BWG), feed conversion ratio (FCR = feed intake: body mass gain) and mortality (MORT) of broilers. Body weight gain was significantly reduced with Cd supplementation. There were significant differences noted between T1, T2 and T3 in BWG at age 0-7 d (P<0.0001), 7-14 d (P<0.0001) and 14-21 d (P<0.0001) and between T2 and T3 compared with T1 at ages 21-28, 28-35, and 35-42 respectively. On the other hand, no significant differences were noted between T3 and T2 at age 21-28 (P<0.0001), 28-35 (P<0.0001) and 35-42 (P<0.0001).

The different treatments significantly affected FCR at different ages from 0-42 days as shown in Table 17. Cadmium supplementation significantly increased feed conversion ratio at age 7-14 d (P<0.0001) and 14-21 d (P<0.05). Significant differences were noted between T1 and T3 but not T2 at age 0-7 d (P<0.0049). In addition, T1 was significantly different compared with T2 and T3 at age 14-21

d (P=0.0110), 21-28 d (P=0.0715) and 28-35 d (P=0.0588), respectively. Furthermore, there wasn't any significant difference between all treatments on FCR at age 35-42 d (P=0.7823) (Table 17).

Table17: The effect of different treatments on BWG, FCR and mortality of broiler chickens

Parameters	**Mean ± SE**			**P-value**
	T1	**T2**	**T3**	
0-7	110.1±3.9 [a]	90.1±2.0 [b]	74.0±0.6 [c]	<.0001
7-14	284.1±3.2 [a]	178.8±4.9 [b]	81.6±2.1 [c]	<.0001
14-21	877.08±7.631 [a]	549.28±17.070 [b]	398.93±7.865 [c]	<.0001
21-28	463.7±5.7 [a]	339.5±23.0 [b]	310.2±10.3 [b]	0.0001
28-35	516.1±6.4 [a]	433.3±19.0 [b]	418.4±11.9 [b]	0.0013
35-42	568.2±33.8 [a]	531.3±18.5 [ab]	462.4±15.0 [b]	0.0341
0-7	1.32±0.04 [b]	1.42±0.02 [b]	1.55±0.03 [a]	0.0049
7-14	1.30±0.00[c]	1.70±0.04 [b]	2.27±0.02 [a]	<.0001
14-21	1.40±0.00 [b]	2.00±0.17 [a]	1.90±0.09 [a]	0.0110
21-28	1.70±0.000 [b]	1.92±0.08 [a]	1.80±0.05 [ab]	0.0715
28-35	1.80±0.00 [b]	1.92±0.04 [a]	1.80±0.04 [b]	0.0588
35-42	1.95±0.10	1.95±0.08	1.87±0.06	0.7823
0-7	0.00±0.00	0.00±0.00	1.67±1.67	0.4053
7-14	0.00±0.00	3.32±3.32	0.00±0.00	0.4053
14-21	0.00±0.00	1.67±1.67	1.67±1.67	0.6224
21-28	0.00±0.00	0.00±0.00	0.00±0.00	0.000
28-35	1.77± 1.77	0.00±0.00	0.00±0.00	0.4053
35-42	1.77±1.77	0.00±0.00	0.00±0.00	0.4053

Means within a row with different superscripts are different at P<0.05

There were significant differences between T1, T2 and T3 in FC from 0-42 days (P<0.0001) (Table 18). Similarly to FC, WG at age 0-42 days was significant difference in all treatments (P<0.0001). Both parameters (FC and WG) at age 0-42 were higher in T1 compared to T2, compared to T3. On the other hand, mortality didn't show any significant difference during the experiment in all treatments (Table 18). Moreover, FCR results at age 0-42 d revealed no significant difference between T2 and T3, but only compared to T1 (P<0.0047).

Table18: The effect of different treatments on FC (g), WG (g), FCR and mortality of broiler chickens throughout the experimental period (0-42 days).

Parameters	**Mean ± SE**			**P-value**
	T1	**T2**	**T3**	
FC 0-42	3950.6±20.7 [a]	3402.5±60.7 [b]	2839.4±58.8 [c]	<.0001
WG 0-42	2384.7±26.4 [a]	1813.8±57.0 [b]	1549.6±34.1 [c]	<.0001
FCR 0-42	1.65±0.028 [b]	1.90±0.057 [a]	1.82±0.025 [a]	0.0047
MORT 0-42	3.32±3.3	5.00±3.18	3.35±1.93	0.8963

Means within a row with different superscripts are different at P<0.05

Body weight (g) and mean feed consumption (g) are presented in Table 19. Except at day 0 where no differences were noted (P= 0.1540), body weight (BW), was significantly affected by the treatments at all time points. It was significantly higher in T1 followed by T2 and then T3 (P<0.0001).

Similarly, mean feed consumption (MFC) was affected by the treatments. It was higher in T1 followed by T2 and then T3 (P<0.0001) for the first six weeks of the experiment. In the last week (35-42 d) MFC showed no significant difference between T1 and T2, but was significantly lower in T3.

Table 19: Mean, Standard error, and P-value of BW and MFC.

Parameters	Mean ± SE			P-value
	T1	T2	T3	
BW 0	40.52 ±0.43	39.65±0.221	40.35±0.20	0.1540
BW 7	150.70 ±4.37 [a]	129.85±2.15 [b]	114.32±0.69 [c]	<.0001
BW 14	434.82 ±7.04 [a]	308.62±7.10 [b]	195.95±1.60 [c]	<.0001
BW 21	877.08±7.63 [a]	549.28±17.07 [b]	398.93±7.86 [c]	<.0001
BW 28	1340.85 ±9.37 [a]	888.80±34.38 [b]	709.18±17.29 [c]	<.0001
BW 35	1857.03 ±12.58 [a]	1322.13±53.33 [b]	1127.58±26.85 [c]	<.0001
BW 42	2425.28 ±26.51 [a]	1853.45±56.88 [b]	1590.00±34.07 [c]	<.0001
MFC 0-7	146.90 ±1.31 [a]	129.25±1.49 [b]	114.10±0.31 [c]	<.0001
MFC 7-14	373.92 ±1.02 [a]	301.42±8.80 [b]	187.15±6.53 [c]	<.0001
MFC 14-21	624.48 ±9.08 [a]	468.15±10.62 [b]	380.83±19.31 [c]	<.0001
MFC 21-28	784.98 ±10.27 [a]	647.48±21.03 [b]	552.18±14.89 [c]	<.0001
MFC 28-35	923.53 ±3.35 [a]	827.98±16.44 [b]	749.33±21.71 [c]	0.0001
MFC 35-42	1096.85 ±15.04 [a]	1028.20±51.93 [a]	855.80±9.31 [b]	0.0013

Means within a row with different superscripts are different at P<0.05

4.7. Discussion

This study was part of a project designed to explore if simultaneous zinc and organic Se feed supplementation can protect broilers from orally occurred Cd toxicity.

In a study by Lazarus et al. (2009a) it was showed that inorganic Se supplementation reduced Cd levels in the liver and kidney of rat pups. On the other hand, Sasakura and Suzuki (1998) indicated that selenoprotein P may be involved in the formation of a complex binding Cd. Jihen et al. (2008) indicated that Se and Zn have stronger positive effect in reducing Cd toxicity and accumulation in the liver than each one alone. De Haan et al. (1996) indicated that this effect is a state between the first and the second step of the enzymatic antioxidant pathway by examining the concentration of the MDA (malondialdehyde) as an indicator of changes in lipid peroxide (LPO) and the activities of antioxidant enzymes such as CAT, GPx, CuZn SOD, and the CuZn SOD/GPx compound activity ratio which it is an indicator of the balance status in this state.

Many studies indicated that the Cd produces ROS which can induce oxidative damage. Moreover, Cd was shown to decrease the activity of SOD, CAT and GPx (Hassoun and Stohs, 1996). Sarkar et al. (1995) indicated that Cd can negatively affect lipids and produce LPO. Cd increased the LPO in liver as a result of increased formation of free radicals as well as reduced SOD, CAT and GPx activities (Patra et al., 1999). Decreased levels of these enzymes, SOD, CAT and GPx may occur by direct interaction between Cd and Zn in the enzyme molecule. In other words, Cd seems to occupy Zn binding site in CuZn SOD resulting in an inactive form of the enzyme (Bauer et al., 1980). Lazarus et al. (2006) indicated that the reduction in activity of GPx might be due to depletion of Se by Cd or due to the formation of a chemical complex between Cd and Se at the active site of the enzyme (Gambhir and Nath, 1992)

Jurczuk et al. (2004) indicated that in the presence of Cd in the organism the resulted decreased levels of Fe in blood, liver and kidney may negatively affect CAT expression. Moreover, CAT contains Fe in its active center; the decreased activity of the enzyme in the liver of rats exposed to Cd might be a result of Fe deficiency. On the other hand, Se and Zn are essential elements in all plants, animals and human and can act to protect organs from Cd toxicity in different tissues (Chowdhury et al., 1978).

The present study showed that Se and Zn can work synergistically to protect organs from the harmful effects of Cd toxicity. This result is similar to the results by Yiin et al. (1999) who demonstrated a protective role of Se in Cd induced lipid peroxidation in rat testes.

To our knowledge, this study is the first one attempting to investigate a possible protective role of simultaneously supplemented organic Se and Zn against Cd toxicity in four different tissues, namely liver, kidney, breast and blood. Additionally, this study showed a correlation between Se, Zn and Cd and a correlation of those elements with others such as Ca, Cu, Fe, Li, Sb, and V (Table 15).

In conclusion, the present study revealed the accumulation of 17 essential, probably essential and toxic, elements in four different tissues. Moreover, showed were the correlations between Se, Zn and Cd with Ca, Cu, Fe, Li, Sb and V.

As mentioned above, this research was part of a project investigating the possible positive effect of simultaneous Se and Zn supplementation against induced Cd toxicity on broiler performance. As Wlosotowski et al. (2005) indicated, Cd absorption and accumulation inside animal tissues are related to the dose, age, gender, species and nutritional states but also to the level of other essential elements which can act positively against Cd toxicity.

Our results showed that there were significant differences in BW and BWG at all ages among all treatments. In addition, we indicated that there weren't any significant differences in mortality due to Se and Zn supplementation, which was in agreement with the results of Cantor et al, (1975a, b) and Edens et al. (2001).

Cantor et al. (1982) indicated that Se concentration was high in breast in turkey poults fed selenomethionine upon nutritional muscular dystrophy. Similar to these results were our results. Analysis of data showed that the Se and Zn concentrations were high in breast too.

Concerning the targets of Cd toxicity and the sites of antagonistic interactions between Zn and Cd, Xiao et al. (2002) indicated that in the kidney Zn can reverse and reduce Cd induced oxidative stress. Moreover, in kidney the protective effect of Zn can arise despite an increase in Cd content (Jacquillet et al., 2006). Oishi et al. (2000) indicated that high level of Cd concentration has been found in kidney in animals fed with Cd. Cadmium renal retention was accompanied by an increase in Zn concentration and a highly positive correlation was demonstrated between Cd and Zn. The results of our present study were in line with these results. Data analysis showed that the highest concentrations of Cd were measured in kidney followed by the liver and breast. Similarly, Se and Zn were mainly accumulated in liver and kidney. Moreover, Cd, Se and Zn were significantly positively correlated in all examined tissues.

Although productive parameters did not indicate positive effects after Zn and Se supplementation, our hematological data and mortality results demonstrated that higher levels of Zn and Se may ameliorate some of the negative effects of Cd toxicity since there were no significant negative effects found upon Cd contamination.

In conclusion, Zn and Se supplemented in chickens' diet can probably help protecting from the negative effects of Cd toxicity at least at the levels of Cd tissue accumulation. Furthermore, reduced was the accumulation of other toxic elements such as As, Cr and Pb. Further studies should be conducted to elucidate the mechanism of the obtained results of Cd toxicity.

Part C

General Conclusions

General Conclusions

The main objective of this research was to investigate, i) whether organic Se added to the feed could protect the broilers from Cd toxicity, after Cd was added to the chickens' diet in different concentrations, ii) the effect of Cd in chickens' health and performance, iii) the benefits of organic Se and both organic Se and Zn against the toxicity of administered Cd and iv) the accumulation and correlation of these elements (Cd, Se and Zn) and 14 further elements (toxic and essential) in different tissues namely liver, kidney, blood and breast.

It was revealed that the level of Cd present the diet determines the accumulation of other elements namely Cu, Fe, Sb, Se and V. While low levels of added Cd only affected the level of accumulated Cd, high levels of added Cd increased the concentration of Cd, Sb, V and reduced that of Se and Fe. In addition, highest accumulation of Cd was found in kidney and liver the two target tissues of Cd in the body, which is known to be bound with metallothionein. Furthermore, Se concentration was reduced in the body which maybe related to its use in Se-Cd complexes, or for antioxidant protection against the induced oxidation by Cd.

On the other hand, it has been shown that V strongly induces the synthesis of MT. High levels of Se and Cd in chicken feed significantly increased tissue levels of V which may be able to act protectively by increasing the level of MT in the corresponding tissues.

Furthermore, this research showed that there were strong correlations between Se and Cd prior to the application of the statistical model but weak positive correlation in the post model application. Accordingly, there are many studies showing strong correlation, negative correlation and non correlation between Se and Cd.

In the present work, Zn showed different accumulation trends in both experiments against Cd toxicity. In the first experiment, Zn was not correlated with Cd, but this was significantly different in the second experiment, probably because of the strong Zn-Se positive correlation. These two elements may have synergistically cooperated against Cd toxicity.

As mentioned before, Zn an integral part of the enzyme CuZn superoxide dismutase (CuZn-SOD). Further, Zn is involved in cell membrane stabilization and MT synthesis, which can act against Cd toxicity in the body. Zinc supply in conditions of exposure to Cd can partially protect against Cd-induced oxidative stress and restore CuZn-SOD activity in red blood cells of rats while Se and Zn can have a cooperative effect in the protection against Cd-induced structural damage in the liver but not in

the kidney of rats. Moreover, the accumulation of Cd in the body can be affected by different factors such as type of tissues, the age of animals, gender, species, nutritional status and by dietary intake of other elements that may interact with Cd.

It should be mentioned that it was not clear whether the differences in elements' tissue accumulation were related to the ratio of Cd, Zn and Se present in the diet, to the formation of complexes or other indirect interrelations that need to be elucidated.

Chickens' performance such as body mass and feed consumption were decreased by the high level of Cd (50 ppm and 100 ppm) in the present work. While there wasn't any significant difference in those performances at low (10 ppm) Cd level.

Previous studies with simultaneous Cd and Se supplementation revealed that although Se could not counteract for Cd-induced decreased performance, it improved other examined parameters such as tissue structural damages, antioxidant defense and apoptosis.

The present work indicated that Se (and Zn) can help against the negative effects of Cd but cannot neutralize all of them indicating that more research is needed to establish the optimum level of inclusion or use of these essential elements in conjunction with other antioxidants.

Finally, this work suggested some possible study extensions. Such extensions may include: metallothionein expression and the gender of birds recruited for the experiments. How MT can protect the body after high administration of Cd with or without Se and Zn is an aspect of importance. Furthermore, blood pH and its alterations may be a factor which could be studied in order to clarify and understand how the body is affected by the Cd and other toxic metals. Blood can provide a fast and easy way to investigate heavy metals in the body.

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