2012

IN OVO SELENIUM (SE) INJECTION OF INCUBATING CHICKEN EGGS: EFFECTS ON EMBRYO VIABILITY, TISSUE SE CONCENTRATION, LIPID PEROXIDATION, IMMUNE RESPONSE AND POST HATCH DEVELOPMENT

Lizza M. Macalintal
University of Kentucky, lmmaca2@uky.edu

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Dr. Austin H. Cantor, Major Professor

Dr. David L. Harmon, Director of Graduate Studies

ABSTRACT OF DISSERTATION

Lizza M. Macalintal

The Graduate School
University of Kentucky
2012

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ABSTRACT OF DISSERTATION

An abstract of a dissertation submitted in partial fulfillment of the
requirements for the Degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

by

Lizza M. Macalintal

Lexington, Kentucky

Director: Dr. Austin H. Cantor, Associate Professor of Animal Sciences

Lexington, Kentucky

2012

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Studies were conducted to investigate the effects of *in ovo* injection of selenium (Se) either as seleno-methionine (Se-Met) or sodium selenite (Na_2SeO_3) into the yolk of incubating eggs on tissue Se concentration, embryo livability, lipid peroxidation, immune response and growth performance. When white-shelled eggs were injected with 0.1ml of solutions providing 0, 2.5, 5, 10 or 20 μg Se/egg, no detrimental effects on embryo viability at 20 days of incubation were noted. The effects on tissue Se concentrations suggested that Se-Met and Na_2SeO_3 were metabolized differently by the chick embryo. In a subsequent study using injection doses up to 60 μg/egg, a greater linear response in tissue Se was obtained with Se-Met, compared with Na_2SeO_3 ($P < 0.01$). Minimal changes in heart and breast muscle Se concentrations were noted above the 40 μg dose when Na_2SeO_3 was used ($P > 0.05$). In a study with broiler eggs, injection doses of 0, 2.5, 5, 10, 20 and 40 μg Se/egg were used. Se-Met or Na_2SeO_3 at doses up to 40 μg Se/egg had little effect on embryo viability. Injecting Se-Met resulted in greater tissue Se accumulation than Na_2SeO_3 at 20 days of incubation. In another study with broiler eggs using injection doses up to 40 μg Se/egg, Se-Met injection resulted in higher hatchability, reduced lipid peroxidation in the lung and heart muscle of the embryos after 20 days incubation and higher Se concentrations in heart and breast muscle of hatched chicks through 7 days and in lung through 21 days of growth. In a feeding trial with broiler breeder hens, adding 0.3 mg/kg of Se as Se yeast or Na_2SeO_3 to the diet improved tissue Se status at hatching of progeny chicks. Taken together, these results indicate that injection of Se into the yolk of incubating eggs may be useful for enhancing Se status during embryonic and early post-hatch development. Therefore, the improvement in Se status using this method in conjunction with dietary Se supplementation of breeder hens would be much greater than with only using dietary supplementation.

Key words: *in ovo* injection, selenium, embryo, lipid peroxidation, sheep red blood cell

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IN OVO SELENIUM INJECTION OF INCUBATING CHICKEN EGGS: EFFECTS ON EMBRYO VIABILITY, TISSUE SE CONCENTRATION, LIPID PEROXIDATION, IMMUNE RESPONSE AND POST HATCH DEVELOPMENT

By

Lizza M. Macalintal

Dr. Austin H. Cantor

Director of Dissertation

Dr. David L. Harmon

Director of Graduate Studies

April 26, 2012

(Date)

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Jeremiah 29:11-14

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CHAPTER 1: INTRODUCTION

Selenium's role in animal health and nutrition was first recognized in the 1930's as a toxic principle that occurred in feed ingredients. Consumption of seleniferous plants from certain areas in South Dakota has caused major problems to poultry raisers as hatchability and growth performance were affected. Franke and Tully (1935) first described the toxic effects of selenium in chickens grown in certain localities in South Dakota, USA. Farmers raising these chickens complained of low chick hatchability, hatched chicks had greasy down that never became fluffy and when higher hatchability was obtained, chick livability was lower. Tully and Franke (1935) attributed the inhibited growth of chicks and reduced egg production from toxicants that naturally occur in grains grown from "alkalied" areas. In a related study, Franke and Tully (1936), induced the teratogenic effects in chick embryos using selenite injected into the aircell with a reported lethal dose of 50% (LD_{50}) at 0.7 ppm. However, even at a low concentration of 0.01 ppm, embryonic abnormalities were observed. In 1958, Gruenweld described the histologic changes that occurred in embryos <5 d of incubation hatched from hens fed a diet containing 45% wheat and supplemented with Se at 17 ppm. The primary visible effect in the embryo was necrosis of the cells in the brain, spinal cord, optic cups and lens vesicles and in the mesenchyme of the limb buds and somites of the tail region.

Subsequent studies included injecting incubating eggs using different routes of administration, Se compounds and stages of development. The toxic effects of Se included high mortality and developmental defects. Depending on Se compounds used, route and days of injection, these experiments led to different estimates of the LD_{50}. Aircell injection resulted in higher death than the yolk sac route. Organic (Se-Met, selenocysteine) as well as inorganic (selenite, selenate) forms of Se were used as Se sources, however, Se-Met was observed to be more toxic than Na_2SeO_3 (Palmer *et al*, 1973). Dietary addition of Se at toxic levels to hen diets resulted in decreased in hatchability, egg weight and egg production. Eggs that did not hatch after 21 days showed embryos with head and neck enlargement (Ort and Latshaw, 1978).

Se toxicity occurs when the body's ability to excrete excess Se is compromised. However, "since the physiological basis of selenosis has been defined and the biochemical indicators have not been identified, it is difficult to distinguish levels of Se that are safe and beneficial from those that are potentially harmful to health" (Ralston *et al*, 2008).

Although Se was first associated with toxicity, in 1950's, the importance of selenium in the diet was elucidated when it was deemed essential in the prevention of liver necrosis in rats. Hence, its essentiality was established. This was further strengthened in the 1970's when Se was found to be an essential component of the enzyme glutathione peroxidase (Rotruck *et al*, 1973). Since the essentiality of Se in the animal diet had been established, more research was then focused on the requirement to prevent deficiency effects. Deficiency of Se in the poultry diets has shown to cause several pathological conditions that can impact growth and development.

Normally, Se is added to the animal's diet using inorganic Se (Na_2SeO_3 or Na_2SeO_3). However, in 2000, the United States Food and Drug Administration (FDA) approved the use of Se yeast as an organic Se source of which the major Se component is Se-Met. It has been reported that Se is readily transferred from breeder hens to the eggs and, thus, to the embryo (Cantor *et al.*, 1974; Paton *et al.*, 2002). However, the amount of Se that can be derived from the hen's diet is limited, because the maximum level of dietary Se supplementation is limited to 0.3 ppm by the FDA.

One unique way of introducing nutrients to the incubating embryo is through *in ovo* injection. Studies have shown that *in ovo* injection of nutrients, such as carbohydrate, proteins, vitamins and amino acids as well as vaccines, can enhance growth and development of the embryo, improve energy status, promote early gut development and improve immune status, as well as alleviate the stress of hatching (Foye *et al*, 2007; Tako *et al*, 2005; Gore and Qureshi, 1987; Ohta *et al.*, 2001; Johnston *et al.*, 1997; Sharma and Burmester, 1984).

The objective of this research was to study the effects of *in ovo* injection of high levels of Se either as Se-Met or Na_2SeO_3 in fertile eggs at 10 d of incubation on tissue Se concentrations in both embryos and hatched chicks, embryo viability, lipid peroxidation

and immune responses during post-hatch development.

CHAPTER 2: REVIEW OF LITERATURE

Historical Background of Selenium

Records of selenium-related illnesses in animals date back to Marco Polo's travels to western China (1271–1295) (Spallholz, 1994). Polo attributed the loss of hooves and hair in horses to the consumption of poisonous grasses that researchers now believe contained high concentrations of Se. However, the element Se was not discovered until 1817 when Swedish scientist, Jons Jakob Berzelius isolated and identified it "in the sulfur obtained by sublimation from the iron pyrites of Fahlun" (Turner, 1835). Se is named after the Greek goddess of the moon, Selene, because chemically it is similar to the element tellurium, a name derived from the Latin *tellus* meaning Earth.

In the 1800s, "alkali disease" was documented in grazing livestock that had access to alkali seeps and high salt concentrations in the U.S. upper Midwest (*e.g.*, Nebraska, South Dakota). Symptoms included emaciation, loss of hair and hooves, signs of anemia, liver cirrhosis, and skeletal erosion. Not until 1935 was the underlying cause of "alkali disease" identified as Se toxicity. Franke and Potter (1934) proved this association by experimentally reproducing similar symptoms in rats by feeding them selenite or selenate. Separately, Moxon (1937) reported another disease affecting the nervous system of cattle and sheep, which he called "blind staggers," since affected animals tend to exhibit unsteady gait. He further suggested that these animals had ingested plants that were accumulators of high concentrations of Se.

Selenium was considered to be a toxic element until 1957, when Schwarz and Foltz showed it was an essential trace element based on experimental evidence that liver necrosis in rats can be prevented when their diets contain brewer's yeast but not torula yeast. Brewer's yeast differentially contains Se, which together with vitamin E and cysteine, can prevent liver necrosis, Se being what is now known as Factor 3. In separate studies Schwarz *et al.* (1957) and Patterson *et al.* (1957) also showed that Se can prevent exudative diathesis (ED) in chicks. In addition, Se therapy was shown to prevent diseases like muscular degeneration, which occurs naturally in lambs (Schubert *et al.*, 1961) and calves (Muth *et al.*, 1958; Schubert *et al.*, 1961). In relation to these, Thompson and Scott (1970) concluded that Se is essential for growth of chickens independent of its function

as a substitute for vitamin E. Concurrently, Rotruck *et al.* (1973) and Flohé *et al.* (1973) discovered the selenoprotein glutathione peroxidase (GSH-Px), the activity of which is dependent upon tissue Se uptake. This discovery confirmed the essentiality of Se in animal and human health. To date more than 20 selenoproteins (Arthur and Beckett, 1994) have been discovered, each of which is tissue specific and dependent on Se availability.

Extensive studies have been conducted to investigate the metabolic effects of Se in both animals and humans, but it was not until 1974 that the FDA approved the inorganic Se (sodium selenite) for use as a dietary feed supplement for poultry and livestock (Leeson and Summers, 1991). In 2000, the FDA approved the use of organic selenium in the form of Se yeast (Sel-Plex®, Alltech Inc., Nicholasville, KY), the primary Se component of which is seleno-methionine (Se-Met).

Biochemistry of Selenium

Selenium is classified as a metalloid, having the characteristics of both the non-metal and metal elements. With an atomic number of 34 and a molecular weight of 78.96, it is a member of Group VIA on the periodic table together with oxygen, sulfur, tellurium and polonium. Of these elements, sulfur is very similar to Se both in its chemical and physical natures (Table 2.1, Figure 2.1), nevertheless *in vivo* there are differences and one cannot always substitute for the other (Shamberger, 1983). For example, in mammals Se tends to be reduced, whereas sulfur is usually oxidized. In terms of its relative acidity, hydrogen selenide (H_2Se) is more acidic than hydrogen sulfide (H_2S). Under physiologic pH, cysteine is protonated, whereas selenocysteine (Se-Cys) is usually in a dissociated or deprotonated form.

Currently, both inorganic and organic Se are widely studied and used as feed supplements. Substances are classified as inorganic if after combustion of live tissues the remaining ash is predominantly in the form of oxides, carbonates, or sulfate (Underwood and Suttle, 1999). Inorganic minerals were traditionally added to feed, but because of the antagonism that exists between salts and other components of the digesta its bioavailability is often compromised leading to lesser absorption of specific minerals.The

organic form of minerals implies that they are bound principally to an organic substrate (Radcliffe *et al.*, 2007). Organic Se in the form of Se-Met (Combs and Combs, 1986) is the natural form of Se normally ingested by animals from plant-based ingredients. Furthermore, Se-Met is the predominant Se species present in the organic Se yeast.

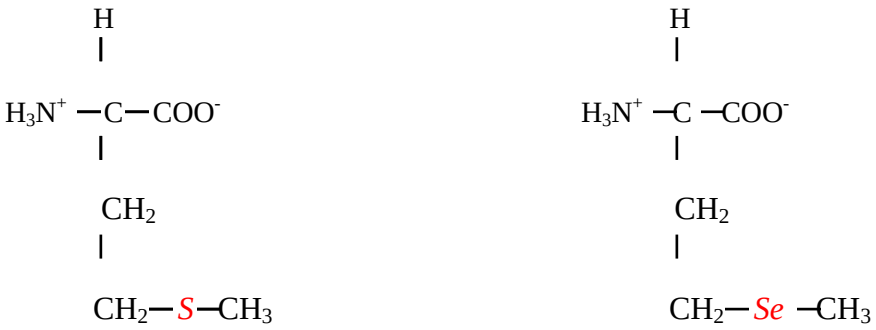
Selenium exists in four oxidative states: selenide (Se^{-2}), elemental Se (Se^{0}), selenite or selenous acid (Se^{+4}, SeO_3^{-2}) and selenate or selenic acid (Se^{+6}, SeO_4^{-2}). These valance states are important because they affect both selenium's solubility in water and its absorption in the intestine.

Table 2.1. Comparison of sulfur and Se compounds.

	Sulfur	**Selenium**
Oxidation State	---------- Inorganic forms ----------	
-II	Hydrogen sulfide (H_2S)	Hydrogen selenide (H_2Se)
0	Elemental sulfur (S^0)	Elemental selenium (Se^0)
IV	Sulfite (SO_3^{-2})	Selenite (SeO_3^{-2})
VI	Sulfate (SO_4^{-2})	Selenate (Se_4^{-2})
	---------- Amino acids forms ----------	
-II	Methionine	Selenomethionine
-II	Cysteine	Selenocysteine

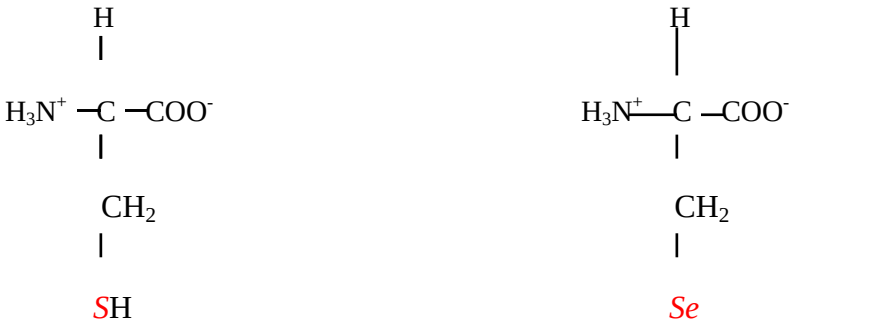
Adapted from Jacques, (2001).

Figure 2.1. Structure of sulfur- and methionine – amino acids.



Methionine

Seleno-methionine



Cysteine

Selenocysteine

Adapted from Schrauzer (2000), with modifications.

Selenoproteins

Selenium can be incorporated in either an enzyme or protein subunit (*i.e.*, as a selenoenzyme or selenoprotein). The name "selenoprotein" is reserved for those Se-containing proteins in which the Se has a specific biochemical function. This is in contrast to Se-containing proteins in which the sulfur amino acid were replaced by their Se analogs. During the synthesis of selenoproteins the UGA (the stop codon code in protein synthesis) is recognized as an insertion codon for the Se-Cys residue (McKenzie *et al.*, 2002) instead of a stop codon to terminate protein synthesis. The biochemical

function of selenoprotein is dependent on the type of enzyme with which it is associated. There are thought to be between 30 to 50 selenoproteins; of these more than 20 have been identified (McKenzie *et al.*, 2002). Of those identified, the most characterized are glutathione peroxidase (GSH-Px, glutathione: H_2O_2 oxidoreductase, E.C. 1.11.1.9.) (Mills, 1957), thioredoxin reductase and iodothyronine deiodinase (Table 2), which play roles in antioxidant defense, redox cycle and hormone regulation.

Table 2.2. Some mammalian selenoproteins and their proposed functions.

Selenoproteins		Tissue, cellular distribution, functions
Glutathione peroxidase (GPx)		
Cytosolic GPx (GPx I)	H_2O_2+2GSH →2H2O +GSSG	Many tissues and cell, cytosolic
Plasma GPx (GPx II)	H_2O_2+2GSH →2$H_2$2O +GSSG	Plasma, kidney, GIT, thyroid,
Gastrointestinal GPx (GPx III)	H_2O_2+2GSH →2$H_2$2O +GSSG	Gastrointestinal tract, antioxidant
Phospholipid hydroperoxide GPx (GPx IV)	ROOH +2GSH → ROH +2GSSG +H_2O	Many tissues and cells, testes; cytosolic and membrane
Epidiymal-androgen-related protein GPx (GPx V)	H_2O_2+2GSH →2H_2O +GSSG	Structural form of spermatozoa
Olfactory GPx (GPx VI)	H_2O_2+2GSH →2H_2O +GSSG	Embryo and olfactory epithelium
Iodothyronine deiodinase		
Types I and II		Catalyzes the conversion of T4 to 3,5,3' T3
Types I and III		Catalyzes the conversion of T4 to 3,5'3' reverse T3
Thioredoxin reductase		Multiple roles associated with its role as part of dithiol-disulphide oxidoreductase system
TrXR1, TrXR2 and TRβ		

Adapted from Kohrle *et al*., (2005) and Jacques (2001).

Selenium Metabolism

Although both organic and inorganic Se are metabolized in different ways, they both first must be converted in the erythrocytes to selenide (H_2Se), which supplies the active Se form used in selenoprotein synthesis (Sunde *et al.*, 1997). Several factors can affect the metabolism of Se: its chemical form; the presence of sulfur, arsenic, metals, microorganisms, vitamin E; and Se status (NRC, 1983).

Se-Met follows the methionine pathways of metabolism (McConnell and Cho, 1965) and thus is kinetically similar (Wolffram *et al.*, 1989) in that methionine crosses the luminal membrane via the electrogenic Na^+-dependent neutral amino acid transport system. Animals cannot synthesize Se-Met, so it must be acquired through diet (Schrauzer, 2000).

Recently, using mouse liver, it was established that the Se-Met is directly metabolized to monomethylselenol by the **γ**-elimination enzyme (Nakamuro *et al.*, 2000). This mechanism may explain why Se-Met is metabolized quickly to form trimethylselenonium (TMSe). Mainly found in cereals and forage crops, Se-Met can replace methionine (Met) because $tRNA^{Met}$ cannot distinguish Met from Se-Met (Schrauzer, 2000). Accordingly, the importance of Se-Met lies in the fact that even though both selenite and selenate can be used for selenoprotein formation it is only Se-Met that can be can directly incorporated into bodily proteins (Figure 2.2).

There are two pathways involved in the catabolism of Se-Met: 1) transulfuration (Beilstein and Whanger, 1992), or 2) transamination-decarboxylation (Mitchell and Benevenga, 1978), more commonly known as transmethylation. Transulfuration involves the production of Se-Cys via the selenocystathione and subsequently glutathione (GSH), thus leading directly to GSH biosynthesis from homocysteine. To rid the biologic system of homocysteine, the transulfuration pathway is upregulated such that GSH synthesis is enhanced while transmethylation is downregulated (Matte, 2007). Previous studies show that an increase in homocysteine levels can be associated with certain pathologic conditions.

Selenite is reduced to selenide by selenoglutathione and glutathione selenopersulfide, whereas Se-Met and Se-Cys are metabolized to hydrogen selenide by the enzyme β-lyase (Ip, 1998). In the liver, Se-Cys is metabolized to serine and selenide. Selenide can then be used as follows: 1) to synthesize selenoproteins, or 2) methylated to dimethyl selenide (DMS) and TMSe ion. The TMSe form can either be exhaled (via the lungs) or excreted (via urine) (Fairweather *et.al.,* 2010). Since Se-Met can be directly degraded to methylselenol (CH_3SeH) by the enzyme β-lyase, this metabolic process can be achieved by the presence of the vitamin B_6-dependent enzyme, cystathionine β-synthase.

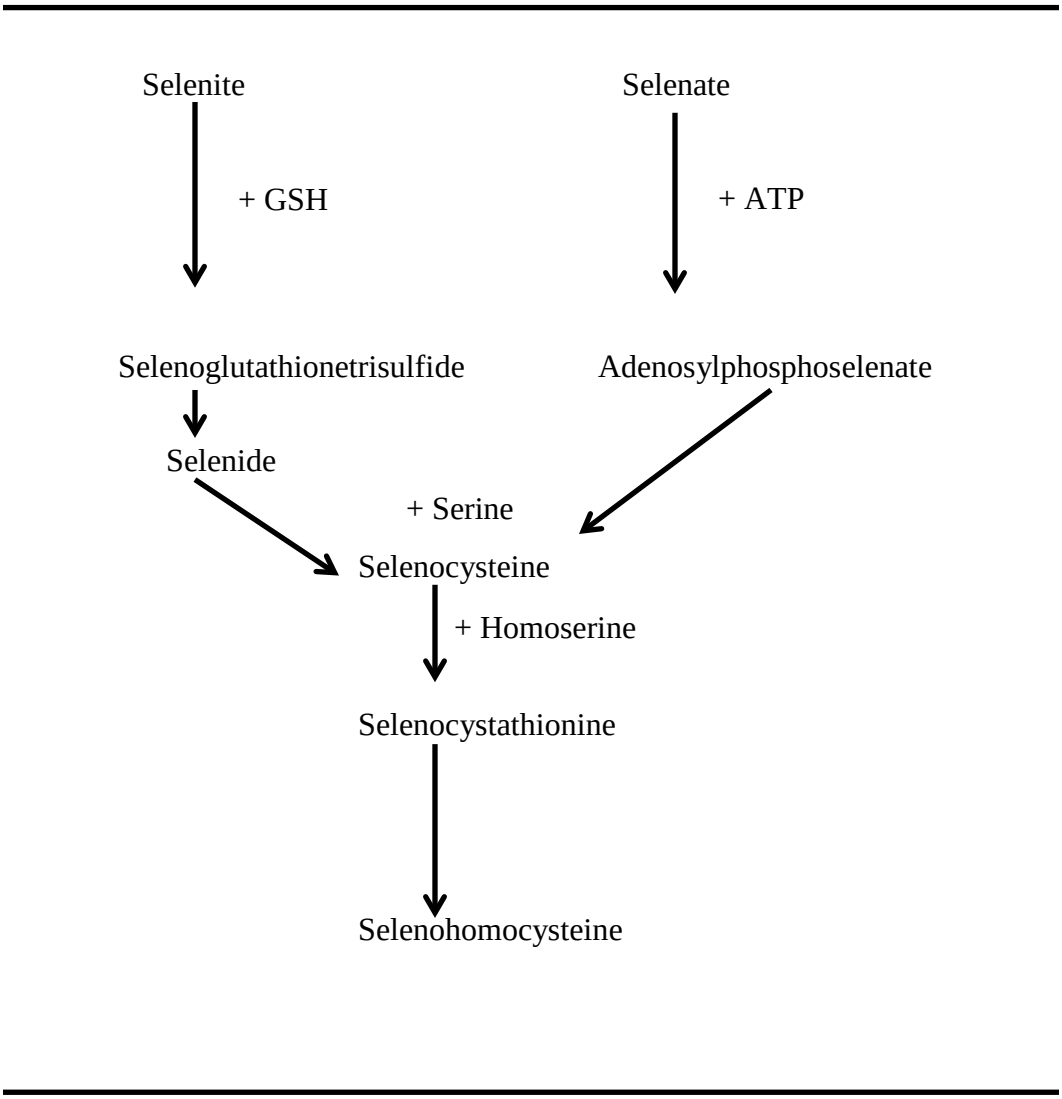


Figure 2.2. Seleno-methionine biosynthesis in plants, marine algae and brewer's yeast.
Adapted from Schrauzer (2000) (Copyright ©2000 American Society for Nutrition).

Selenium Absorption

The amount of Se that is absorbed and how the body utilizes it depends on whether the form of Se is organic or inorganic (Mahan, 1994). There are three possibly methods of Se absorption: 1) inhalation, 2) ingestion, and 3) absorption through the skin. Although to date the metabolism of Se in birds has not been thoroughly studied (Wilson *et al.*, 1997), in general, ingested Se is thought to be absorbed in the small intestine, particularly the duodenum, via the Na^+-dependent neutral amino acid transport system (Vendeland *et al.*, 1994) and is then distributed to almost of cells in the body. As demonstrated using a rat model, apparently no absorption occurs in the stomach (Whanger *et al.*, 1976). Absorption however, is dependent not only on the chemical species and amount ingested (Shamberger, 1983) and Se status of the animal (Daniels, 1996), but also on the absorption site (*i.e.*, duodenum). By using everted rat intestine, the isolated brush border membrane vesicle (BBMV) fraction revealed differences in kinetics between Se forms. For example, response to selenite was found to be curvilinear, whereas the response to Se-Met was linear. Selenite was present at concentrations almost 37-fold higher than selenate (Vendeland *et al.*, 1994) and Se-Met is 14-fold higher selenate In a study using mallard ducks injected with selenious acid, it was shown that visceral tissues responded to injection in a triphasic manner (*i.e.*, a rapid rise, a decline, and another rise after 24 h), whereas Se concentrations in the brain increased continuously from 15 min to 24 h post-injection (Wilson *et al.*, 1997). In this same experiment, the ovaries tended to accumulate Se more so than did visceral organs. The investigators concluded that ovaries may preferentially absorb selenium, which could potentially lead to reproductive abnormalities or embryo toxicity. Furthermore, they suggested the human placenta could possibly protect against toxicity, a mechanism that is not found in egg-laying species.

Study of the uptake of Se in the form of selenate in the brush border membrane of human placenta has shown that the transport system is similar to that of the sulfate pathway (*i.e.*, Na^+-independent), which is also inhibited by chromate, molybdate, tungstate and sulfate (Shennan, 1988). Thus, Se is a competitive inhibitor of sulfate in the microvilli of human placenta.

In terms of Se form, Se-Met has been found to be more efficiently absorbed than the inorganic selenite, which is absorbed via passive diffusion (Wolffram, 1999). Swanson *et al.*, (1991) found that ^{74}Se from selenite was 84% absorbed compared to 98% for Se-Met after subjects consumed a daily diet containing 87 μg dietary Se, 94 g fat, 304 g carbohydrate and 98 g protein. The Se from inorganic sodium selenite, sodium selenate and calcium selenate were shown to be absorbed at the same rate in chicks in trials using high levels of inorganic Se supplementation, with the kidney having the highest Se concentrations (Echevarria *et al.*, 1988a,b).

The presence or absence of other nutrients affects the uptake or absorption of Se. Mykkanen and Wasserman (1989) concluded that vitamin-D deficiency could impair selenite absorption in chicks based on analysis of duodenal BBMV in rachitic chicks and vitamin-D-treated rachitic chicks. Similarly, high doses of vitamin A (Combs, 1976) or vitamin C (Combs and Pesti, 1976) can also lead to increased intestinal uptake of Se.

Transport and Storage

In general, trace minerals have specific carriers once they are effluxed from the basolateral membrane of enterocytes to the general circulation, which ensure these ions do not exist in free form. After Se is absorbed it is carried in the plasma (Buescher *et al.*, 1960) bound to plasma proteins (McConnell and Levy, 1962). From there it enters the different tissues of the body. Although Se can be found in almost every cell in the body, its concentration varies according to the type of tissue as well as the level of Se intake (Schamberger, 1983).

The uptake of Se-Met represents binding whereas the uptake of selenite or selenate represents both binding and transport into the vesicle (Vendeland, 1994). The selenite transport system has kinetics similar to that of zinc (Zn) in that it is sodium independent and occurs through simple diffusion (Wolffram, 1999).

Bioavailability

Since selenium's discovery as an essential nutrient the majority of nutritional studies, especially those involving poultry, have focused on the dietary effects of sodium selenite. More recently, organic selenium in the form of Se yeast has been the subject of study because there are indications that the organic form of Se tends to be more bioavailable compared with the inorganic form. Bioavailability is the amount or percentage of a substance that passes from the gastrointestinal (GI) tract to the plasma under normal physiologic conditions (Wolffram, 1999). Although both Se-Met and selenite are converted to selenide, the method by which they are metabolized into Se-Cys for incorporation to GPx differs. Se-Met can be incorporated directly into tissue proteins and stored which helps elevate the amount of Se-Met that is retained (Henry and Ammerman, 1995). In contrast, although selenite is highly absorbed is not well retained in body tissues since its passive mode of absorption is similar to that of a mineral (Wolffram, 1999)

The bioavailability of selenium has been studied in the prevention of exudative diathesis (ED) in chicks (Cantor *et al.*, 1975a). Using different feedstuffs and Se compounds, after 12–21 d of feeding the bioavailability of eight plant-based Se sources ranged 65–210% compared with Se from animal origins, which ranged 8–24%. The bioavailability of selenium from the following compounds was as follows: selenate (74%), seleno-DL-met (39%), and elemental Se (7%). They further demonstrated that the protection against ED is highly correlated with GPx level in chicks fed with selenium. At 6 and 7 d post-Se-Met supplementation, there was a lack of positive correlation between Se-Met and plasma GPx activity. Selenite or Se-Cys was found to be more effective in preventing ED than Se-Met. However, in a related study (Cantor *et al.*, 1975b), Se-Met was found to be more protective against pancreatic degeneration in chicks compared with selenite or Se-Cys. Cantor *et al.* (1974) concluded that the bioavailability of selenium is determined by the ability of the chicks to utilize selenium for GPx activity and that selenite is better utilized for GPx activity than Se-Met, and that Se-Met and selenite have different modes of action in chicks with respect to ED and pancreatic fibrosis. In another

study using turkeys, Cantor and Tarino (1982) concluded that Se availability from selenite exceeded that of Se-Met based on plasma GPx activity.

Excretion

Selenium can be excreted from the body via three routes: 1) respiratory, 2) urinary, and 3) intestinal. When selenite is metabolized to hydrogen selenide, the TMSe ions derived are excreted in the urine, whereas DMSe (a volatile compound) is expelled by the lungs (Nakamuro *et al.*, 2000). Generally, the amount of selenium eliminated is dependent on the amount and form of dietary Se and its biologic interactions with other elements (Schamberger, 1983), such as cadmium, arsenic, copper, silver, manganese, lead, mercury, thallium, tellurium, vanadium and bismuth.

Distribution of Selenium

A. Plants

Selenium is taken up from the soil by plants as selenite, selenate or organic selenium (Johnson *et al.*, 1967) and incorporated during the synthesis of Se-substituted analogues of thiomolecules (Ralston *et al.*, 2007). The predominant form of Se in plants is Se-Met (Schamberger, 1983). The Se concentration in plants is dependent on several factors including soil pH, oxidation rate of artificial fertilization and rainfall (Surai, 2006) as well as sulfur, calcium, phosphorus and nitrogen concentrations (Schamberger, 1983). Selenium is delivered to chloroplasts in the leaves through the xylem system. Once Se is assimilated it is processed via the sulfur pathway into organic compounds (Surai, 2000).
Different plants have different capacities for incorporating Se from the soil and are categorized accordingly: 1) Se accumulators, 2) secondary Se accumulators, 3) non-Se accumulators (Surai, 2006). The Se accumulators are those that are associated with Se toxicity symptoms observed in the initial stages of Se research. These plants, grown in seleniferous soils, tend to accumulate selenium in leaves and stems. Secondary Se accumulators have high Se concentrations even if they are grown in soils with low-to-medium-Se content. Meanwhile, the non-Se accumulators are plants with less than 25 ppm Se and which do not accumulate Se in excess of 100 ppm even if they are cultivated in highly seleniferous soils (Terry *et al.*, 2000). In an experiment using duckweed, Se

from selenite tended to accumulate three times faster than from selenate (Peterson, 1967). In a translocation experiment conducted by Gissel-Nielsen (1976), it was shown that after30 min more than 15% of Se was translocated from selenite compared with more than 80% from the amino acid fraction, which he surmised was most likely in the form of Se-Met. Overall, plant-based Se tends to accumulate in animal tissues whereas selenite does not (Cantor *et al.*, 1974). Plant-derived Se in forages and grains is mainly composed of organic forms such as Se-Met and Se-Cyst although Se-Met represents 50% of naturally occurring Se (Olson and Palmer, 1976). In animals, the Se component of Se-Met must be released from the amino acid complex and the Se converted to selenide before it can be converted to Se-Cyst (Olson and Palmer, 1976).

B. Soil and Geographic Location

In general, the concentration of minerals in plants reflects the mineral content of the soil. When soil mineral concentration is low, either plants will have lower mineral concentrations in the seed/grain or plant growth will be reduced, or both. The concentration of Se in soil varies but is thought to average about 0.01 ppm (Bohn *et al.*, 1985). Kubota *et al.* (1967) studied the relationship of soil and Se in forages and feed crops in the United States. They categorized the states according to soil Se level (Figure 2.4). Soils in South Dakota, Nebraska, Kansas and Colorado tend to have higher soil Se concentrations (2–10 ppm) because they are derived from cretaceous shales, whereas soils from the Great Lakes, Northwest and Southwest have low Se concentrations (<0.05 ppm) because they are derived from volcanic deposits or well-washed coastal deposits. The regional distribution of soil Se is important because it correlates to the Se content of local forages and grains. Notably, low-Se areas, such as Illinois, Ohio and Indiana, are where most of the corn and soybean meal used by the poultry industry is sourced. Likewise, some areas in the southeastern United States where the soil Se is either variable or low also have high concentrations of poultry producers. A survey conducted by Cantor *et al.* (1997) on Se levels in corn from 10 states (Delaware, Illinois, Indiana, Iowa, Kentucky, Maryland, Minnesota, Nebraska, Ohio and Virginia) and soybean meal grown in 18 states (Alabama, Arkansas, Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Minnesota, Mississippi, Missouri, Nebraska, North Carolina, Ohio,

South Carolina and Virginia) revealed that state averages for Se levels in corn ranged from 0.024–0.428 ppm, whereas for soybean meal the range was 0.06–0.80 ppm.

C. Tissue Se Distribution

After absorption, tissue retention of Se is dependent on several factors including type of tissue/organ; amount and form of dietary Se; length of feeding time; and animal Se status, species, and age. Tissue concentration is also greatly affected by the amount of Se supplemented in the breeder diet; hence maternal plane of nutrition is of significance to embryo Se status.

In the avian egg (using 6 terrestrial bird types), the Se content was found to be as follows yolk > chalazae > internal viscous albumen > external liquid egg white (Golubkina *et al.*, 2006), which is in agreement with previous studies showing that Se is more concentrated in egg yolk (Paton *et al.*, 2002; Edens and Sefton, 2002; Cantor, 1997).

As for eggshells, studies have shown that it too contains Se although not in proportions as high as those of egg contents. The egg shell might also be an additional source of Se for the growing embryo (Surai *et al.*, 2004), which is in agreement with the study conducted by Golubkina and Papazyan (2006) in which the concentration of egg shell Se decreased ($P < 0.01$) as the embryo developed in two groups of hens fed diet containing 0.3 ppm Se as sodium selenite or Se yeast. The different sources of Se differed in the amount of Se that accumulated in the egg hell as well as in the eggshell membrane.

As previously mentioned, Se is carried to the liver and kidney after its absorption. In an experiment conducted by Aspite and Atlavin (1994), 4 h after injecting a radioisotope of selenium (^{75}Se) concentrations of Se in chicken tissues were as follows: duodenum > liver > kidney > feathers > jejunum and ileum > spleen > pancreas > blood > breast muscle. In contrast, Surai *et al.* (2006) supplemented quail diet with 0.5 ppm organic selenium and found the following Se tissue distribution: kidney > liver > heart = lung > breast > leg muscle > brain. In another study, Se-deficient 4-month-old Wistar rats were injected with 0.13 µg Se (^{75}Se) in the vena caudalis on d 1 and d 5 and later showed preferential accumulation of Se in the brain, endocrine and reproductive organs compared with tissues such as heart, muscle and liver (Behne *et al.*, 1988).

Selenium Deficiency

The essentially of Se in animal diet was reported by Schwarz and Foltz (1957). Since then studies on Se deficiency in poultry have also been reported that confirm the importance of dietary Se (Edens, 2002, 2001,1996; Edens *et al.*, 2002; Combs and Combs, 1984; Combs and Scott, 1977; Cantor *et al.*, 1975a,b; Latshaw *et al.*, 1977; Latshaw and Osman, 1974; Gries and Scott, 1972). Furthermore, Se deficiency when coupled with low vitamin E can be responsible for an array of poultry diseases including ED and nutritional encephalomalacia (Noguchi *et al.* 1973; Combs and Hady, 1991).

Certain metabolic disorders can also be linked to deficiency since Se is a component of the enzyme iodothyronine deiodinase (DI) types I, II and III. Se-dependent enzyme type I functions in the deiodination of T_4 to the metabolically active T_3 (Becket *et al.*, 1987). In a study by Jianhua *et al.* (2000), Se-deficient chickens showed significant reductions in T_3 and elevated T_4 levels compared with Se-supplemented chickens. Likewise, 5'deiodinase activity was lower. When a monoiodinase inhibitor such as iopanoic acid was added to the diet, T_3, T_4 and 5'hepatic DI activity were diminished to levels comparable to those of the control, leading to reductions in weight gain, which suggests that dietary Se tends to increase the rate of skeletal muscle breakdown. This finding is in agreement with Brown *et al.* (1981) who concluded that Se supplementation in rats increased muscle protein breakdown leading to heavier body weights (BW).

Selenium deficiency has also been documented in humans, especially in regions where soil Se concentrations are poor leading to low concentrations of Se in staple grains and human diet. For example, in China Se deficiencies in people are associated with Keshan disease, which is characterized by multifocal necrosis and replacement fibrosis of the myocardium, which can lead to heart failure (Chen *et al.*, 1980; Li *et al.*, 1985).

Selenium and Immunity

Dietary Se plays an important role in all aspects of the immune system (Arthur *et al.*, 2003). Selenium is a known component of GPx, which removes lipid hydroperoxides and hydrogen peroxide and converts them to water and non-radical alcohols. GPx compounds are found in the different cellular compartments (*e.g.*, cytosol, extracellular space, cell membranes). Therefore the antioxidant properties of selenium are present throughout the cell as well, participating in immune signaling.

Swain *et al.* (2000) reported that feeding broiler chicks with a combination of 150 IU/kg vitamin E and 0.1 ppm Se as Na_2SeO_3 significantly increased antibody production against Newcastle disease vaccination as measured by ELISA titer. Furthermore, Se plus vitamin E combination diets compared with basal diets showed a significant increase in leukocyte migration inhibition. This study shows the importance of Se as well as vitamin E in increasing the immune status of broilers. Other studies have had similar results (Singh *et al.*, 2006; Raza *et al.*, 1997).

Levkut *et al.* (2009) suggested that the effect of organic Se in increasing the concentration of circulating T and B cells is important in restoring the leukocyte subpopulation in chicks fed a deoxynivalenol (DON)-contaminated diet, leading to increases in cellular phagocytic activity. They found increased concentrations IgM^+, CD^{44+}, CD^{45+} and MHCII+ peripheral blood lymphocytes in broilers chicks fed a diet containing DON + Se yeast (corn with a background of 0.5 mg DON/kg feed) and Se yeast supplemented control diet compared with a control diet or mycotoxin-contaminated diet without Se yeast addition. Additionally, Se-fed broilers challenged with coccidia exhibited enhanced immune response (Colnago *et al.,* 1984) through higher blood packed cell volume than chickens fed a basal diet. Leukocytes were retained at the site of infection, but were not destroyed. That they were not destroyed by the infection confirms their role in protecting the host against the pathogenic effects of the parasite.

Leng *et al.* (2003) conducted an experiment comparing the effect of Na_2SeO_3 and organic Se on immune response of layers and reported that organic Se was two times better than selenite in eliciting an immune response, as evidenced by an increase in CD^{3+}, CD^{4+} and CD^{8+} surface cell markers. Surface cell markers display the T cells to the

antigen-presenting cells. The CD^{3+} marker is present in all T lymphocytes; CD^{4+} is a marker for T helper cells; whereas CD^{8+} is a marker for cytotoxic T cells. Furthermore, based on the results of the study, they concluded that dietary organic Se enhances the immune status of the birds via increased mobilization and ability of immune cells to respond to infection.

Selenium was also reported to stimulate the transformation of T lymphocytes into cytotoxic cells in mice (Kiremidjian-Schumacher and Roy, 1998). Three groups of mice were fed a diet containing Se at normal, supplemented or deficient levels: torula yeast (0.2 ppm Se), selenite (2 ppm Se) and Se deficient (0.02 ppm Se). Se supplementation significantly increased the ability of resting lymphocyte to respond to mitogen stimulation; conversely, Se deficiency decreased mitogen stimulation. In another study, in vivo activated macrophages from Se-supplemented mice showed an enhanced capacity to destroy tumor cells (58.16 vs 49.17%); this cytotoxic ability may be related to the chemopreventive properties of selenium (Kiremedjian-Shumacher *et al.*, 1992). In vitro testing showed that the neutrophils from Se-deficient mice rats and cattle can phagocytose pathogens, but the neutrophils of Se-supplemented animals can phagocytose as well as kill pathogens.

Another method of measuring in vivo cell-mediated delayed hypersensitivity reaction is through intradermal administration of phytohemagglutinin (PHA) in toe webs of birds. In brief, Biswas *et al.* (2006) reported that dietary Se at 0.5 and 1 ppm increased antibody response to inoculated sheep red blood cells (RBC) and mitogen response to PHA measured as foot web index at 4 weeks compared with the basal diet (0.2 ppm Se) in Japanese quail; Se treatments had no effect on performance. In contrast, Gowdy and Edens (2003) reported that broilers fed organic Se showed less T-cell-mediated wing web reaction to PHA compared with selenite-fed birds.

Selenium and Poultry

Selenium can be maternally transferred from breeder hens to eggs to progeny. Supplementing poultry diets with inorganic Se, especially in the form of sodium selenite, has been a common practice in the poultry industry, because Se was been shown to be an essential nutrient in poultry. In 1974 the FDA first approved the use of Na_2SeO_3 in swine and poultry to prevent economic losses due to Se deficiency, which were estimated around $82 million (Ullery, 1980; FDA, 1974). In 1978 an amended Se regulation was submitted with the following changes: Se can be supplemented up to 16 weeks of age at a level not to exceed 0.1 ppm for chickens or 0.2 ppm for turkeys (Ullery, 1980). In 1981, the FDA approved the supplementation of selenium at 0.1 ppm to laying hen diets (FDA, 1981). Ullrey reviewed the basis for this regulation of Se supplementation in animal diets in 1992. In 2000, the FDA approved the use of organic selenium in the form of Se yeast (Kelly and Power, 1995) in poultry (FDA, 2000; 2002). Se yeast contains a high level of Se-Met, a naturally occurring form of organic Se found in plant-based feed ingredients.

Selenium and Maternal Transfer

The developing embryo is highly dependent upon the nutrients transferred from the dam via the yolk. After the egg is laid, the fertile egg is isolated from additional nutritional influences other than simple gaseous exchange (Noble, 1986). The contribution of yolk lipids to aerobic metabolism through β oxidation is critical to the embryo's successful development (Surai, 1999). All of the energy needed by the growing embryo is contained in the lipid fraction of the egg contents, which includes the yolk (McNabb, 2000). Therefore maternal nutritional status during egg development is of critical importance.

Gaseous exchanges and high metabolic rates during embryonic development can lead to the production of reactive oxygen species (ROS) free radicals (Halliwell, 1994). These free radicals can cause cellular damage leading to peroxidation. Antioxidants play an important role in combating these substances and providing protection to cells and the developing embryo overall (Surai *et al.*, 1996). Antioxidants include vitamin E, vitamin

C, and three antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and Se-containing GPx.

Selenium Toxicity and the Chick Embryo

Early embryo nutrition is essential for successful incubation in conjunction with appropriate environmental factors and egg quality. Thus, the nutritional status of eggs, *i.e.*, type and concentration of nutrients passed on by the dam to the developing embryo, is important before oviposition since development of chicks occurs outside of the maternal body. During embryonic development, the yolk is the primary source of nutrition. Therefore, the presence of energy and nutrients such as amino acids, vitamins and macro or microminerals and other nutrients should be optimum for maximal growth and development. One such important micro mineral is Se. The effects of Se on transfer from breeder hens to progeny have been previously studied (Cantor and Scott, 1974; Paton *et al.*, 2002; Surai, 2000; Pappas *et al.*, 2005; Pan *et al.*, 2007, Latshaw and Osman, 1975) and its positive influence on post-hatch chicks is well documented. Cantor and Scott (1974) showed that dietary selenium in hen diet does not lead to excessive Se uptake in eggs and is required for growth and protection of progeny. Pre-hatch access to selenium might help retard peroxidation brought about by the heightened ROS concentration during the hatching period, as indicated in previous studies on early nutrition (Foye *et al.*, 2007; Tako *et al.*, 2005), which revealed that pre-hatch access to nutrients as β-hydroxy-β-methyl-butyrate (HMB) and Zn methionine contribute to enhanced jejunal nutrient uptake and digestion as well as improved gut functionality in contrast to sham controls. Thus, early feeding may help offset some constraints related to hatching.

Previous studies have shown that yolk Se concentrations are highest between d 10–15 of incubation (Paton *et al.*, 2002; Surai, 2000) when dams are supplemented with Se from either organic or inorganic sources. During this period, when graded levels of Se (0.1- 0.3 Se ppm as Na_2SeO_3or Se yeast) were supplied to the dam, the net effect for the extra embryonic Se levels was significantly higher from Se yeast compared with Na_2SeO_3 or no Se due to increased overall Se status of the egg.

Likewise, embryonic Se concentrations were greatest Se-yeast-supplemented hens. Therefore, both the source and dose of selenium greatly affect the Se content in the egg, embryo or extra-embryonic fractions.

In terms of the pro-oxidant property of Se, a study by Ort and Latshaw (1978) in which layer diet was supplemented with graded levels of Na_2SeO_3 (0, 0.1, 1.0, 3.0, or 5.0 mg/kg Se) revealed that up to 5 mg/kg Se was nontoxic to laying hens, *i.e.*, there were no significant effects on egg weight, hatchability or egg production. However, when the layer diets were supplemented with 7 or 9 mg/kg Se, egg weight and hatchability declined ($P < 0.05$). Furthermore, the addition of 9 mg/kg Se in the diet significantly reduced ($P < 0.05$) egg production. The only visible defect in embryos that did not hatch by 21 d of incubation was an enlargement of the head and neck region attributed in part to fluid retention and in part to abnormal tissue development.

Franke and Tully (1935) first described the toxic effects of Se in chickens grown in certain localities in South Dakota, USA. Farmers raising these chickens were complaining of low chick hatchability, hatched chicks had greasy down that never became fluffy and when higher hatchability was obtained then chick livability was lower. Based on their experiments, eggs obtained from the affected farms had 107 / 139 eggs that failed to hatch. They reported that embryos exhibited the following malformations such as, upper beak stubby or missing, beak crossed, enlargement of head or neck, no eyes, and one eye. These low hatchability and embryo anomalies were probably due to the supplementary feeds given to the hens. In a subsequent experiment, Tully and Franke (1936), attributed the inhibited growth of chicks and reduced egg production from toxicans that naturally occur in grains grown from “alkalied” areas. In a related study, selenite was directly injected into the aircell and it induced teratogenic effects on the embryo, the LD50 was reported as 0.7 ppm but even a dose as low as 0.1 ppm produced these embryonal defects (Franke and Tully, 1936). These defects were similar to those seen when laying hens in their earlier studies. Franke *et al.* (1937) further studied the effects of Se poisoning on hatchability. Toxic grain such as corn, wheat or barley containing about 15.15 ppm Se resulted in hatchability that decreased to zero but resumption of normal diet after 7 days hatchability was restored.

In 1958, Gruenweld described the histologic defects that occurred embryos <5 d of incubation) hatched from hens fed a diet containing 45% wheat and supplemented with selenium at 17 ppm. Visible primary effects of Se supplementation transmitted from the hen to the embryo was necrosis of the cells in the brain and spinal cord; in the optic cups and lens vesicles, in the mesenchyme of the limb buds and in the somites of the tail region. These changes could explain the physical anomalies described in the previous woks of Franke and Tully (1935).

One of the first studies on Se injection to chick embryos was that of Franke *et al.* (1936) who reported that the LD_{50} (lethal dose) for eggs incubated at d 0 or before incubation to be 0.7 mg Se /kg as Na_2SeO_3. LD_{50} is a common method for measuring substance toxicity and is defined as the dose that kills 50% of the animals exposed – thus, the lower the LD_{50}, the more toxic the substance. Ridgway and Karnofsky (1952) studied the effects of 56 metals including selenium on the chick embryo, using two routes of inoculation (*i.e.*, yolk and chorioallantoic membrane (CAM)) using a volume between 0.05–0.2 ml. They estimated the LD_{50} for Se to be 0.03 mg Se/egg from selenate (H_2SO_4) when injected at 4 d of incubation via the yolk sac. This study produced fewer chick abnormalities (10–20%) in the surviving embryos at 18 d of incubation compared with those obtained by Franke *et al.* (1936). On the other hand, when Halverson *et al.* (1965) injected fertile chicken eggs with 0.1 ml solution that contained Se as sodium selenite, potassium selenite, sodium selenate or potassium selenate in various concentrations at 14 d of incubation, an LD_{50} of 0.5 µg Se/ml was observed for selenite-Se and an LD_{50} of 1.8–2.0 µg Se/ml was observed for selenate-Se. Chicks hatched from these experiments showed growth depression but no visible signs of abnormalities except for some instances of dorsal swelling of the neck.

Kury *et al.* (1967) injected 1034 White Plymouth Rock fertile eggs with selenious acid (H_2SeO_3) or selenite in saline solution via the yolk sac at 80–86 h of incubation. The concentrations used to inject the fertile eggs ranged from 0.010 to 0.025 mg Se/egg (*i.e.*, 0.2-0.5 ug/g). These embryos were injected at 4 d of incubation and examined grossly at19 d of incubation. Embryos examined at d 19 of incubation showed both malformed

(22%) and normal embryos (78%). In addition, the surviving embryos were anemic, characterized by low red blood count and hemoglobin values.

Palmer *et al.* (1973) concluded that Se-Met is more toxic to the chick embryo when injected via the air cell at a rate of 0.1 ml with the LD_{50} at 0.13 µg Se/ml (levels of Se ranged from 0.0 - 0.4 mg Se/egg). For Na_2SeO_3, the LD_{50} was calculated to be 0.3 µg Se/ml (levels ranged from 0.0 - 0.8 mg Se/egg). These investigators suggested that the higher levels of toxicity exhibited in this study compared with results of Franke *et al.* (1936) and Halverson *et al.* (1965) were attributed to the fact that at 4 d of incubation embryos are at a more critical stage of development than either 0 h (pre-incubated) fertile eggs or 2-wk-old embryos.

Sukra *et al.* (1976) reported that a Se injection between 0.1- 0.2 µg Se/ml as Na_2SeO_3 after a mercury injection improved the survival of the embryos more so than mercury alone. However, in a more recent study, 6-d-old chick embryos that were injected with 0.02 mg Se / embryo as Na_2SeO_3 all died within 48 h after inoculation (Szelezczuk *et al.*, 2004). Table 3.1A and 3.2B list the results of earlier studies involving egg inoculations with selenium.

Most of the studies Se and chickens to date involved the quantification of the concentration or distribution of dietary inorganic or organic selenium in the heart, breast muscle, liver, kidney, spleen, whole blood, serum (Payne and Southern, 2005; Pan *et al.*, 2007), eggs, and whole embryo (Paton *et al.*, 2002). "Selenium has been shown to be efficiently taken up in the intestinal cells as well as the kidneys but the transporter system involved in this uptake has not been identified" (Nickel *et al.*, 2008). Previous studies on brush border membrane uptake of selenium from other species have been inconclusive (Leblondel. *et al.*, 2001; McConnell and Cho, 1965; Vendeland *et al.*, 1992; Wolffram *et al.*, 1989).

In a study by Wilson *et al.* (1997), the kinetics of Se (^{75}Se) in avian species was elucidated. Using 1-year-old female mallard ducks, 0.19–0.22 µg per 920 g bird (0.21–0.24 µg/kg BW) was injected via the wing vein and Se tissue levels were determined 15 min, and 1, 4, 12 and 24 h post-injection. The rate of Se disappearance or appearance was measured in different tissues; over 12 h, Se concentration increased in the visceral organs.

Table 2.3A. Summary of embryo studies on pre-hatch selenium injection.

Reference	LD_{50}, µg /g	Incubation stage (d)	Inclusion rate (mL)	Effects
AIR CELL route				
Franke *et al.*, 1936	0.7 as Selenite	0	0.1	<1 ppm high mortality and abnormalities
Halverson *et al.*, 1965	0.5 as Selenite	14	0.1	Depressed growth and some neck abnormality seen in hatched chicks
	1.8–2.0 as Selenate			
Palmer *et al.*, 1973	0.13 as Se-Met	4	0.1	Se-Met more toxic than selenite
	0.3 as Selenite			
Sukra *et al.*, 1976	0.01–0.02 as Selenite	3, 5, 9, 15	0.1	d 9 at 0.02 and d 15 at 0.01 with 83% chick survival
YOLK SAC route				
Ridgway and Karnofsky, 1952	0.03 as Selenate	4, 8	0.1	10–20% embryo survival at day 18 of incubation
Kury *et al.*, 1967	0.010–0.025 mg as Selenite.	80–86 h	0.02–0.05	Anemia, 72% mortality, 22% malformed embryo at d 19 of incubation
Szelezczuk *et al.*, 2004		5, 9, 15	0.1	Death within 24 h (all embryos)

Table 2.3B. Summary of embryo studies on Se supplementation of maternal diet.

Reference	Inclusion rate mg/kg	Effects on 1-d-old embryos
MATERNAL TRANSFER Route		
Ort and Latshaw, 1978	9 mg/kg Se in layer diet as selenite	Embryos with head/neck enlargement
Hoffmann and Heinz, 1988	10 mg/kg Se as Selenite In mallard diet	0.5 µg Se/g in eggs: Decreased hatching; Edema; Decreased growth; Increased GPx activity
	25 mg/kg Se as Selenite in the diet	1.3 µg Se/g in eggs: Decreased hatching; Edema; Decreased growth; Increased GPx activity; Increased uric acid concentration
	10 mg/kg Se as Se-met	4.6 µg/g in eggs: Malformations and increased GPx and succinate dehyrogenase (SDH) activities
Smith *et al.*, 1988	10 mg/kg Se as Se-Met in maternal diet	0.3 µg Se/g in eggs: Decreased growth (black-crowned night heron) increased hepatic thiobarbituric acid reactive substances (TBARS) concentration

Selenium toxicity has also been documented in other species such as pigs (Goehring *et al.*, 1984; Herigstad *et al.*, 1973; Harrison *et al.*, 1983), lambs (Tiwary *et al.*, 2006; Caravaggi *et al.*, 1970; Fessler *et al.*, 2003; Gabbedy and Dickson, 1969) and ruminants (Kaur *et al.*, 2003; Yaeger *et al.*, 1998). The LD_{50} of selenite for sheep has been reported to be 1.9±1.2 mg Se/kg BW (Caravaggi *et al.*, 1970; Lambourne and Mason, 1969). Intramuscular administration of selenite ranged from 0.45–1 mg/kg BW (Blodgett and Bevill, 1987). In lambs up to 14 weeks of age, an oral ingestion of 1–2.2 mg Se/kg BW as selenite resulted in mortality (Gabbedy and Dickson, 1970). According to the study of Tiwary *et al.* (2006), the target organ for Se toxicity in sheep is the heart, as shown by the high concentration of selenite. The authors further concluded that oral exposure to Se as Se-Met doubled its bioavailability, yet it was slightly less toxic than selenite. The greater tolerance to Se as Se-Met can be attributed to the direct incorporation of Se-Met into non-functional structural protein as a methionine replacement (Panter *et al.*, 1996). Thus, Se from Se-Met is incorporated into organs with a high rate of protein synthesis such as the skeletal muscle, which contains almost 50% of the Se pool (Tiwary *et al.*, 2006).

The effect of Se toxicity in relation to other dietary minerals such as As_2O_5, silver, copper, sulfur, lead sulfate, and mercury has also been studied (Lowry and Baker, 1989; Tatum *et al.*, 2000; Jensen, 1975; Halverson and Monty, 1960; Jensen and Chang, 1976; Donaldson and McGowan, 1989), but is not the subject of this discussion.

Selenium Toxicosis: Mechanism of Action

The exact mechanism of Se toxicosis remains unclear but based on the several *in vitro* studies, selenium, as Na_2SeO_3 appears to act as a pro-oxidant (Moak *et al.*, 2001; Cho, *et al.*, 1999; Stewart *et al.*, 1999; Kithara *et al.*, 1993; Spallholz, 1994).

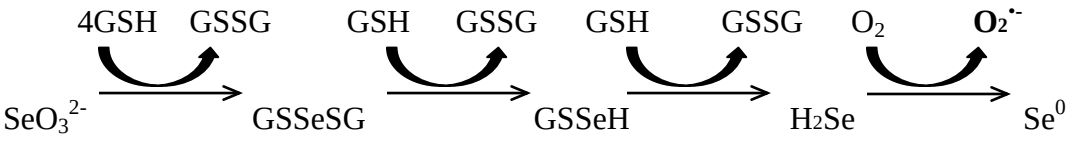
Table 2.4. Ability of Se compounds to generate superoxide *in vitro.*

Superoxide produced	Superoxide not produced
Selenite	Seleno-methionine
Selenium dioxide	Selenate
Selenocysteine	Elemental selenium
Diselenodipropionate	Selenobetaine
Diphenylseleide	Potassium-selenocyanate

Adapted from Mezes and Balogh (2007), Surai (2006) and Spallholz (1994).

The presence of Se compounds intracellularly can produce superoxide *in vitro* via the catalytic oxidation of GSH (Table 2.4). Painter (1941) attributed the toxicity of Se to its ability to react with thiols. Ganther (1968) proposed that the inherent toxicity of Se lies in its interaction with disulfide, a process that produces selenotrisulfide (RSSeSR). Still later, Seko *et al.* (1989) showed that superoxide forms when selenite reacts with glutathione and then with H_2Se (Figure 2.5).

Figure 2.5. Reaction of selenite to produce superoxide.



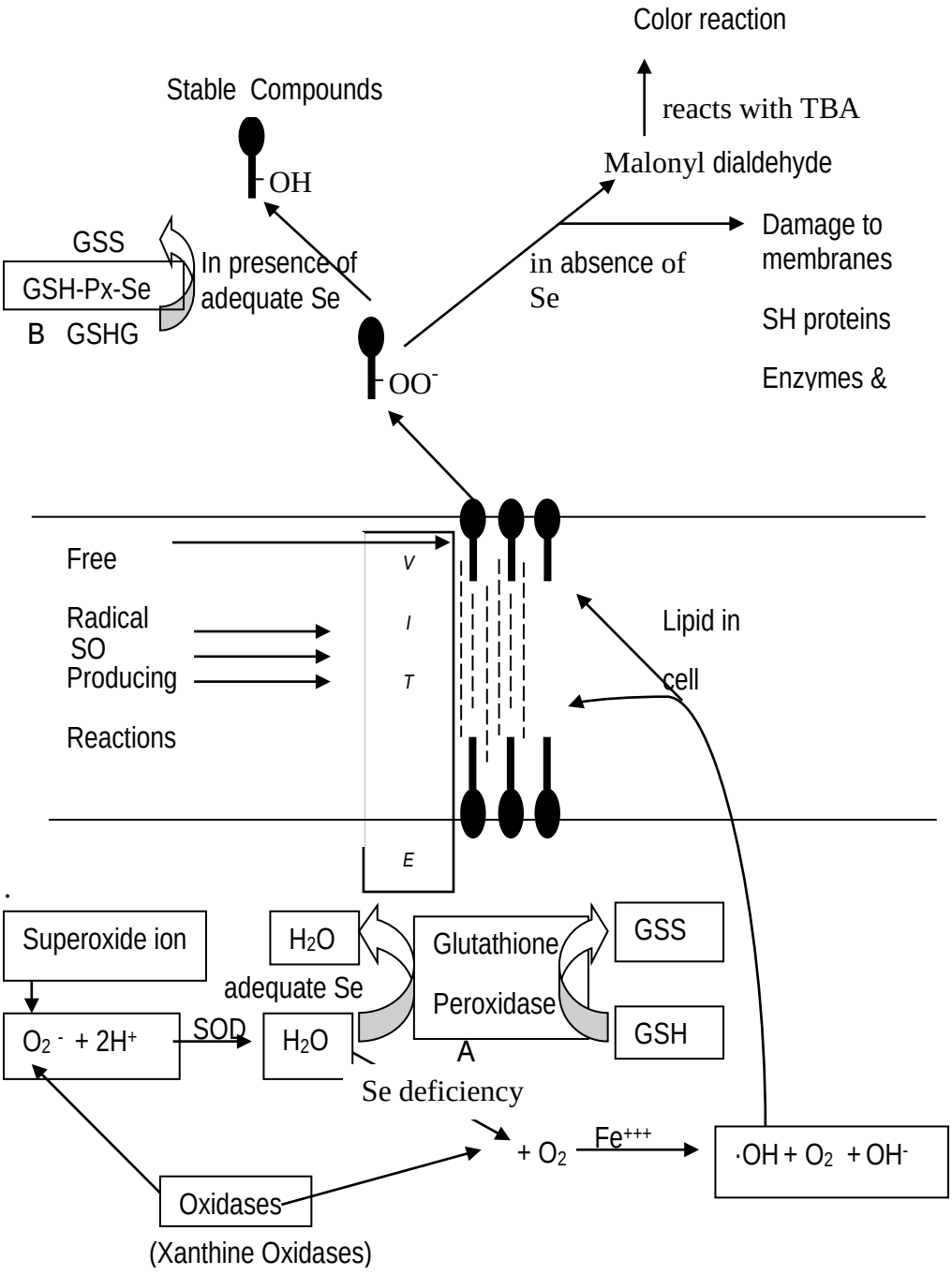
Yan and Spallholz (1993) designed a study whereby they measured the generation of ROS using mammary tumor cell line (HTB123/DU4475). Production of superoxide was measured using lucigenin- or luminal-amplified chemiluminescence. Their study showed a significant increase in the concentration of selenite in the presence of GSH, confirming the findings of Seko. Selenite was shown to react with GSH, and other thiols compounds, to produce superoxide and to a lesser extent hydrogen peroxide. Se-Met and selenate were found to be less cytotoxic to tumor cells than selenite or Se-Cys.

Selenium and Vitamin E

Vitamin E plays an important role in the normal physiologic function of the cellular system as a biologic antioxidant (Tappel, 1973). Vitamin E works in conjunction with other nutrients and endogenous factors that provide protection against harmful effects of ROS, which are produced during normal cellular metabolism or environmental sources (Combs, 1999). One nutrient that works in concert with vitamin E is Se.

The mechanism of Se and vitamin E in the prevention of ED has been studied (Noguchi *et al.*, 1973) to elucidate its role in cellular membrane insult. According to the authors, both vitamin E and Se are necessary for sequestering the lipid peroxides formed in the lipid membranes. When Se and vitamin E were supplemented in chick diet, their mitochondria and microsomes were found to contain both Se and vitamin E, such that lipid peroxidation was completely prevented as measured via thiobarbituric acid reactive substances (TBARS) production, *e.g.* malonaldehyde (Raharjo and Sofos,1993; Noguchi *et al.*, 1973). Conversely, the cellular mitochondria and microsomes of chicks whose diets were depleted of vitamin E and Se showed that these lipid peroxides find their way into all cellular compartments, thus causing damage. Cellular membranes afford little or no protection against peroxidation during hatching when the liver contains very high levels of lipids. In chicks that were supplemented with vitamin E alone (100 IU/kg), peroxidation was almost completely prevented by d 9 however, by d 26 the level of malonaldehyde formed was comparable to that in chicks fed the basal diet. Thus, vitamin E and Se affect the mitochondria in a similar manner. Vitamin E prevents the chain-

reactive auto-oxidation of the lipid membranes, whereas selenium destroys any peroxides present inside the cytosol of the capillary cell. Thus, vitamin E has the ability to neutralize peroxides where they are produced, whereas selenium via the GSH-Px prevents the formation of membrane-destructive hydroxyl radicals ($^{\bullet}$OH) and destroys hydroperoxides (Leeson and Summers, 2001).



Cytosol

Figure 2.6. Mode of action of vitamin E and GSH-Px selenium.
Adapted from Leeson and Summers (2001) and Noguchi *et al.*, (1973).

Mechanism of lipid peroxidation

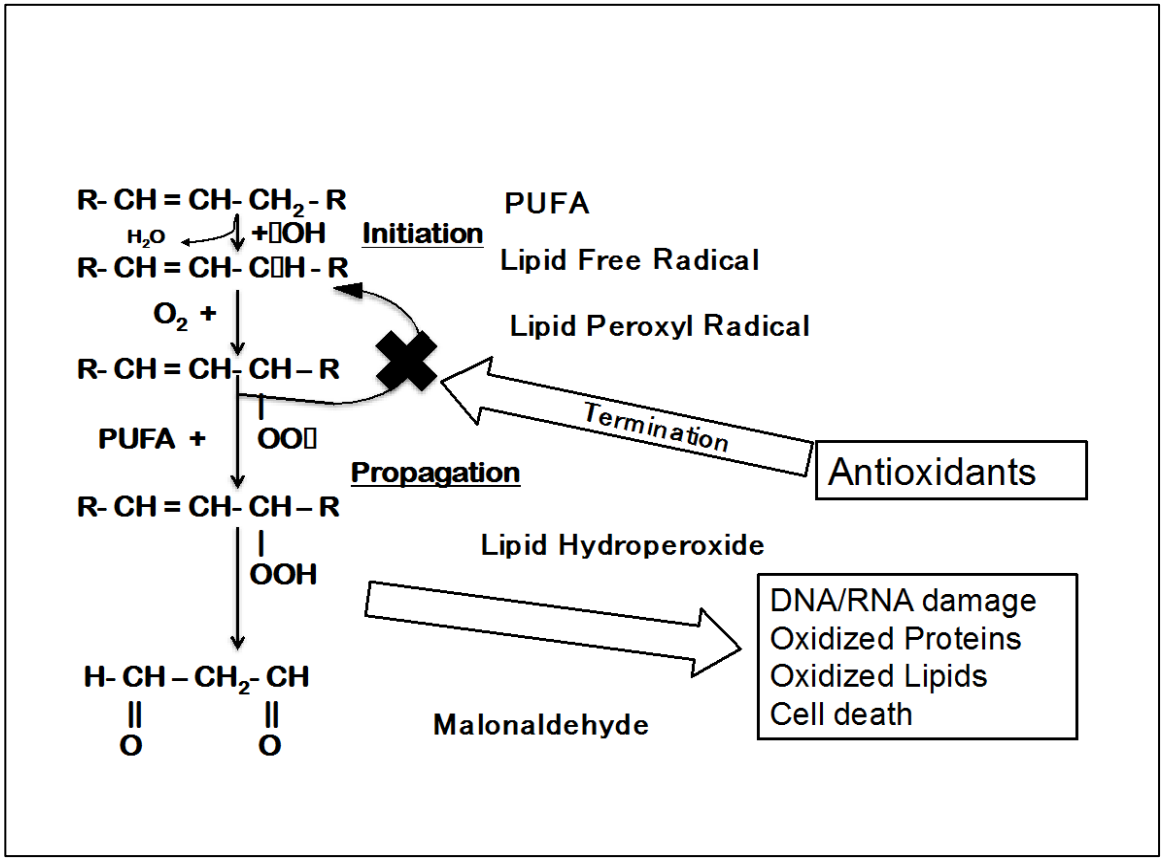


Figure 2.7. Mechanism of lipid oxidation.

Adapted with modifications from: Vickers, (2007) and Combs, G.F. (1999).

Mechanism of Lipid Peroxidation

The biologic membranes that separate cells from their surroundings are chiefly composed of lipids and proteins molecules. Thus, a bilipid layer acts as a barrier to the free passage of inorganic ions and most other charged or polar compounds. The polyunsaturated fatty acids (PUFA) of biologic membranes are susceptible to attacks by free radicals because of the presence of 1-4 pentadiene systems that allow the complete abstraction of a hydrogen atom from one of the $–CH_2$ groups in the carbon chain (Combs, 1999), a process that constitutes the initial stage of lipid oxidation. The abstraction of a hydrogen atom leads to the generation of an unstable C-centered free-radical group, which later undergoes molecular rearrangement to form a more stable conjugated diene.

This conjugated diene is prone to attack by molecular oxygen (O_2), thus generating a peroxyl radical (ROO$^{\bullet}$). When the peroxyl radical abstracts hydrogen from other fatty acids, propagation of the lipid oxidation ensues and this chain reaction continues until the cellular membrane is completely oxidized to hydroperoxides (ROOH) (Combs, 1999), which are then degraded into chain cleavage products in the presence of transition metals. When lipid peroxides are present, the bilipid layer losses its integrity leading to membrane damage and cell death (Padmaja *et al.*, 1997). Thus, when cell membranes are insufficiently protected from oxidative insult, peroxidation of polyunsaturated membrane phospholipids and the inactivation of enzymes can result.

This chain reaction mechanism of lipid peroxidation of the cellular membrane can be prevented by the antioxidant power of vitamin E, which is also known for free-radical scavenging. Vitamin E (L-tocopherol) donates phenolic hydrogens to fatty acyl free radicals, quenching peroxyl groups and preventing the oxidation of PUFA (Table 2.5; Combs, 1999).

Table 2.5. Mechanism of lipid peroxidation and antioxidative reactions.

Lipid peroxidation	Antioxidative reactions
1) HO$^{\bullet}$ + RH → H2O + R$^{\bullet}$	1) α-TH + ROO$^{\bullet}$ → α-T$^{\bullet}$
2) R$^{\bullet}$ + O2 → ROO$^{\bullet}$	2) α-T$^{\bullet}$ + ROO$^{\bullet}$ → Nonradical products
3) ROO$^{\bullet}$ + RH → ROOH + R$^{\bullet}$	

Antioxidant Mechanism

Surai (2003) listed a number of external and internal sources of free radicals that can potentially damage cellular members when produced in amounts greater than the biologic system can control (Table 2.6). Halliwell (1994) proposed three mechanisms explaining how the ˙OH damage can be prevented via repair systems that include both DNA repair, enzymes and proteases, and antioxidant defenses that protect cells from free-radical attack. The presence of natural antioxidants in cells is one way to ensure that cells remain viable in an oxygen-rich environment (Halliwell, 1994). Surai (2003) divided the antioxidant repair system into three levels: Level 1 prevents the formation of free radicals by the removal of free-radical precursors via the SOD, GSH-Px and CAT and metal-binding proteins; Level 2 restricts chain formation and propagation of free radicals via vitamins A, C, and E, carotenoids, ubiquinols, glutathione and uric acid; and Level 3 excises and repairs damaged molecules via the enzymatic actions of lipases, peptidases, proteases, and transferases, among others.

Table 2.6. Sources of free radicals.

Internal	External
Mitochondria	Cigarette smoke
Phagocytes	Radiation
Xanthine oxidase	UV light
Reactions with Fe and other transition metals	Pollution
Arachidonate pathways	Certain drugs
Peroxisomes	Chemical reagents
Exercise	Industrial solvents
Inflammation	
Ischemia and reperfusion	

Adapted from Surai, (2003).

Avian Egg Formation

The complex and highly differentiated parental reproductive cells undergo organizational change leading to the formation of the egg from which the embryo then develops. Egg formation has two distinct phases: 1) growth and maturation of the germ cell, and 2) deposition of the yolk, albumen and membranes (Romanoff and Romanoff, 1963). The yolk is produced in the ovary, which contains the germ cells, whereas the albumen, shell membranes and the shell are supplied by the oviduct. Thus, the formation of the egg starts when the primordial cells migrate into the tissues that are destined to become the ovaries. Egg follicles resemble a bunch of grapes and are composed of oocytes and layers of tissues, which include the vitelline membrane, granulosa layer, theca layers, loose connective tissues, and superficial epithelial tissues. Follicles increase in size as they mature. After stimulation with luteinizing hormone (LH) and follicle-stimulating hormone (FSH) the largest follicle in the cluster ruptures and an egg is released from the ovary into the oviduct, which functions not only as a passageway for eggs but also secretes necessary structural components to complete the egg (Romanoff and Romanoff, 1963). The oviduct is divided into several parts: infundibulum, magnum, isthmus, uterus, vagina and cloaca (Table 2.7). On average, it takes about 24–25 h for eggs to be formed and laid.

Table 2.7. Formation of egg in the laying hen.

Part of Oviduct	Function	Time spent (h)
Infundibulum	Catches yolk, fertilization	0.25
Magnum	Secretes albumen	3.00
Isthmus	Forms the shell membranes	1.25
Uterus	Calcifies shell	20.75
Vagina	Adds cuticle (bloom)	a few minutes
Total		24-25

Adapted from Ensminger, (1992).

Yolk Formation

Yolk formation in chickens begins before the end of incubation and can continue for several months or years. Substances that are needed for yolk synthesis are generally derived from the liver of the laying hen and are then passed to the chick via the blood vessels in the ovary. Yolk formation can be classified into three distinct phases: early, intermediate, and final (Romanoff, 1960); it ceases when the oocytes reached 60–80 µ in diameter (Romanoff, 1960). During this early phase of yolk development, oocytes are encapsulated in a thin membrane or sac called the follicle. A series of cell-restructuring and morphologic changes occur, until the follicular cells increase in height such that they are pseudostratified. At this point, yolk formation takes place peripheral and central areas of the oocyte. Several changes occur in the cytoplasm that leads to increased mitochondrial size. Mitochondria spread out within the cytoplasm and primarily occupy the periphery of the oocyte, whereas fat spheres appear in the cytoplasm. The yolk then collects towards the center of the ovum. Clear vacuole-containing fluid proteins appear underneath the layer of fat spheres when the oocytes are about 1 mm in diameter (Romanoff, 1960).

The intermediate phase of yolk formation soon follows which lasts about 60 d. This is the phase is characterized by slow growth of the oocyte and a gradual increase in globule growth. Finally, a rapid oocyte growth ensues during the final phase of yolk formation, which is about 6–14 d before ovulation. The final phase is also characterized by the appearance of latebra, the concentric white yolk at the center of the alternating ring of white and yellow yolk. After the follicle ruptures and releases the ovum, the very thin clear vitelline membrane, once completely attached to the follicle, detaches from it and totally encapsulates the yolk. The yolk is then released into the oviduct and transported via peristaltic action. Along the way the albumen, shell membrane and shell are added consecutively, and thus the egg is ready to be laid.

Embryo Formation

Fertilization of the egg occurs in the infundibulum when the sperm penetrates the vitelline membrane and directly enters into the germinal disc, which signals the completion of second maturation division of the egg nucleus (Romanoff and Romanoff, 1963) after which both the egg nucleus and spermatozoon undergo distinctive changes to become the female and male pronucleus. In contrast to mammals, in the avian egg, polyspermy occurs. Thus, many male pronuclei are found floating within the cytoplasm. Only that sperm that penetrates the center of the germinal disc will eventually fertilize the egg, leading to a lone male pronucleus. Male pronuclei that do not enter the germinal disc eventually disperse, degenerate, and disappear (Stepinska and Bakst, 2006). Mori *et al.* (1991) suggested that in quails, the center point of the germinal disc contains the highest level of maturation-promoting factor (MPF), whereas the area in which the remaining spermatozoa degenerate and die has no MPF.

The union of the parent nuclei results in the formation of the segmentation nucleus. Segmentation (*i.e*., cleavage) of the cells marks the beginning of embryonic development. Avian eggs undergo meroblastic (partial) segmentation, thus they are classified as telolecithal eggs, in which a relatively large amount of yolk is concentrated at one protoplasmic pole while living cells are confined to the opposite pole (Romanoff and Romanoff, 1963). The first cleavage when egg is in the isthmus. This cleavage leads to the two-cell stage until the cells reach the 16-cell stage when the shell membrane becomes opaque and arrives in the uterus. By this time, the blastodisc has differentiated into central and marginal zones. The central area is separated from the yolk by the segmentation cavity or blastocoele. The formation of the blastoderm occurs when cells are at the 64-celled stage division. Soon after, the marginal area disappears and cellularization of the periblast begins. The blastoderm differentiates into area pellucida and area opaca. It is at the central part of area pellucida, that the primitive streak develops. After about 12 h of incubation the primitive streak has increased in length and there is a marked growth on the posterior direction (medium streak); at 16–24 h the primitive streak is fully developed (definitive streak). The primitive streak regresses after reaching its definitive length in relation to the size of the area pellucida, and the "head-process"

begins (Romanoff and Romanoff, 1963). The primordial embryonic body begins to develop and the egg is then laid. At this time the blastoderm contains about 20,000 cells (Gilbert, 2000). Due to cellular development in mesodermal tissues and the establishment of the notochord (the primitive axis of the embryo), the avian embryo exhibits a distinct anterior-to-posterior gradient developmental maturity (Gilbert, 2000). In brief, cells of the anterior end of the embryo are already differentiating into various organs while the cells of the posterior end are undergoing the gastrula phase.

The formation of primary endoderm (or hypoblast) extends throughout the entire area pellucida and into the region of the germ cell. The blastoderm covers the yolk and differentiates into two layers during gastrula phase. The first layer is the ectoderm which gives rise to the skin, feathers, beak, claws, nervous system, lens and retina of the eye and the lining of the beak. The second layer is the endoderm, which produces the lining of the digestive tract, the respiratory and the secretory organs (Ensminger, 1992). Therefore, the embryo develops within the area pellucida, whereas the cells of the area opaca nourish the embryo.

Extra Embryonic Membranes

Within 48 h of embryo development after fertilization, the extra embryonic membranes begin to develop. Four membranes grow out of the embryo: 1) chorion, 2) allantois, 3) amnion, and 4) yolk sac (Romanoff and Romanoff, 1963). The amnion and chorion arise from the dorsal folding of extra embryonic somatopleure, whereas the yolk sac is derived from the extra embryonic splancnopleure (McGeady *et al.*, 2006). As for the allantois, it is derived from tissues that are transferred from the yolk sac to the hindgut, probably as a diverticulum of the intestine. The allantois rapidly enlarges and occupies a space between the chorion and amnion (McGeady *et al.*, 2006). As the embryo develops, it completely detaches from the extra embryonic tissues except at the umbilicus, where the body wall and intestine do not yet close.

By the fifth day of incubation the yolk sac completely surrounds the yolk (Speake *et al*., 1998). Because the yolk sac is highly vascularized, it produces blood until the hematopoietic system of the embryo is in place, and it absorbs nutrients from the yolk, which it transfers to the developing embryo. Although open communication exists

between the yolk sac and the intestines, no yolk passes to the gut during incubation (Romanoff and Romanoff, 1963). Enzymatic products produced by the yolk sac endoderm are transferred from the endodermal cells to the blood vessels of the yolk sac and then to the embryo. Thus, the yolk sac is mainly responsible for the transfer of nutrients needed for energy and tissue growth (Noble and Cocchi, 1990).

Yolk lipid uptake begins as a rather slow process during the first 2 weeks of incubation; on the third week and final week very rapid uptake ensues. Aside from yolk components, other nutrients enter the yolk through the umbilicus, such as albumin proteins and water during the last 7 d of embryo development. According to Noble (1986), proteins are transported through transmembrane transport, whereas lipids are absorbed via phagocytosis. During the final period of incubation, nutrients enter the embryo via the intestines after the embryo swallows the amniotic fluid. On average the yolk is composed of 48% water, 17% protein, 33% fat, and 2% carbohydrates (Johnson, 2000), however these nutrient distributions are widely affected by egg weight, genetic strain and hen age (Vieira and Moran 1998). With only 2% of the yolk composed of carbohydrates, 90% of the energy needs of the embryo during incubation period are met by the β oxidation of fatty acids in the mitochondria and peroxisomes to produce acetyl coenzyme A (CoA) (Vieira and Moran 1998; Freeman and Vince, 1974). Before the embryo is hatched, the energy produced by β oxidation is mainly used to regulate body temperature (Speake *et al*., 1998), the opposite of what happens during the start of embryo development, at which time most energy is spent on tissue growth rather than maintenance.

The amnion is the fluid-filled milieu where the embryo floats; it acts as a protective cushion and keeps the embryonic tissues from drying out. In the latter stage of embryonic development before pipping, the amniotic fluid, which contains the egg albumin, is swallowed by the embryo for nourishment (Romanoff, 1960) essential to the first days of life since this internalized yolk serves as an energy reserve.

The last of the extra embryonic membrane to appear is the allantois (Romanoff, 1960), which serves as the embryo's respiratory organ as well as the storage area for nitrogenous waste (Smith, 2007). The size of the allantois increases as the size of the embryo increases. As for the chorion, its importance is apparent only after it fuses with

the allantois forming the highly vascular chorioallantois (Romanoff, 1960), which functions in gas exchange.

Table 2.8. Embryonic development during incubation.

Before egg laying	Fertilization, cell division; cell segregation into groups of tissues
Between laying and incubation	No growth Stage of inactive embryonic life
During incubation First day (h) 16 18 20 21 22 23 24	 First sign of resemblance to a chick embryo Appearance of alimentary tract Appearance of vertebral column Beginning of formation of nervous system Beginning of head formation Appearance of blood islands – vitelline circulation Beginning of eye formation
Second day (h) 35 42	 Beginning of heart formation Beginning of ear formation Heart begins to beat
Third day (h) 50 60 62 64 70	 Beginning of amnion formation Beginning of nasal structure formation Beginning of leg formation Beginning of wing formation Beginning of allantois formation
Fourth day	Beginning of tongue formation
Fifth day	Beginning of reproductive organ formation and sexual differentiation
Sixth day	Beginning of beak and egg tooth formation
Eighth day	Beginning of feather formation
Tenth day	Beginning of hardening of the beak
Thirteenth day	Appearance of scales and claws
Fourteenth day	Embryo turns its head towards the blunt end of the egg
Sixteenth day	Scales, claws and beak becomes firm and horny
Seventeenth day	Beaks turns towards air cell
Nineteenth day	Yolk sac begins to enter the body cavity
Twentieth day	Yolk sac completely drawn into body cavity Embryo occupies the entire egg space except the air cell
Twenty-first day	Hatching of chick

Adapted from Ensminger, (1992).

Preparation for Emergence

The chick embryo emerges from its shell after 21 d of incubation. High hatchability rates are achieved when the incubation conditions are optimum. Emergence of the chick begins when internal pipping commences, which is characterized by the embryo breaking the chorioallantoic membrane and the inner shell membrane adjacent to the air cell. Wittmann and Weissenbeck (1980) suggested that the increase in CO_2/O_2 ratio in the air cell at the end of incubation acts as a stimulus for pipping. At this point, the embryo switches from chorioallantois respiration to pulmonary respiration. A great deal of energy from the yolk is utilized in preparing for emergence (*i.e.*, pipping movement, body rotation). Pipping is achieved through the coordinated action of the twitching of the pipping muscle (*muscularia complexus*) located at the base of the back of the neck and the "egg tooth" of the beak. Between 15–19 d of incubation the relative weights of the pipping muscle and liver increase as the result of increased concentrations of glucose, glycogen, and protein in the pipping muscle, a crucial metabolic profile change as the embryo prepares for emergence (Pulikanti *et al*., 2010). The embryo then proceeds to break out of its shell by twisting its leg muscle (Moran, 2007). After the blood vessels that connect the umbilicus to the shell membranes are completely obliterated, the hatching process is complete, and the chick is free.

Early Nutritional Manipulation

There is an increasing interest in ways to improve the developing embryo through early nutritional manipulation, such as *i.e., in ovo* administration of nutrients. This technique is used to enhance early gut development and may abate the challenges chicks encounter during the first few hours post-hatch when delay in feeding or long periods of fasting are experienced. Although the amount of research on supplementing breeder diets with different nutrients has been increasing, perinatal nutrition has also been gaining recognition and is the subject of current studies.

During the early 1980s and 1990s most *in ovo* studies focused on the delivery of vaccines to the developing embryo. Sharma and Burmester (1984) showed that *in ovo* technology could be successful in vaccinating embryos against Marek's virus. These investigators developed the basis of the *in ovo* injection procedures in use today. From

the early work of Uni and Ferket (2003) there has been an increased interest in pre-hatch feeding. To date sources of protein, carbohydrates, vitamins and minerals (Foye, *et al.*, 2007; Tako *et al.*, 2005; Ohta and Kidd, 2001) have been administered to the developing embryo via the amnion, air cell or yolk sac to improve embryo energy status, hatchability, BW and post-hatch performance. In addition, early feeding may help prepare the chick gut transition from yolk-derived nutrition to external diet. Also the enhancement of intestinal transporters due to *in ovo* administration of nutrients, such as HMB, may aid in early gut maturation leading to the increased nutrient absorptive capacity of intestinal cells (Foye *et al*., 2007).

CHAPTER 3: EFFECTS OF *IN OVO* SELENIUM INJECTION ON CHICK EMBRYO VIABILITY AND TISSUE SELENIUM CONCENTRATIONS

INTRODUCTION

Selenium (Se) has been shown to be an essential trace mineral in poultry nutrition. Thus, a dietary Se deficiency in poultry can lead to symptoms such as exudative diathesis (Scott et al., 1957; Schwarz et al., 1957; Patterson *et al*., 1957; Noguchi *et al*., 1973), pancreatic fibrosis (Thompson and Scott, 1969; Gries and Scott 1972), gizzard and heart myopathies in turkeys (Scott *et al*., 1967; Cantor *et al*., 1982b), reduced fertility (Combs, 1994), reduced hatchability as well as reduced egg production (Latshaw and Osman, 1974; Cantor and Scott, 1974). When dietary Se is supplemented to hen diets, it can increase the transfer of Se to eggs (Cantor *et al*., 2000) and consequently to the embryo (Paton *et al*., 2002), and thus potentially prevent conditions associated with selenium deficiency.

Previous studies have also shown that Se injected into the egg is potentially toxic to chick embryos. Franke et al. (1936) reported lethal doses for 50% (LD_{50}) of embryos of 35 µg Se as selenite injected into air cell of eggs before incubation. This dose also led to a high incidence of embryo abnormalities. Ridgway and Karnofsky (1952) found an LD_{50} of 20 µg Se /egg as selenite when injected via the yolk sac at d 4 and 8 of incubation. Halverson *et al*. (1965) performed an air cell injection of selenite at d 14 of incubation and reported an LD_{50} of 25 µg Se/egg. Palmer *et al*. (1973) injected fertile eggs with Se via the air cell at d 4 of incubation and found that Se-Met was more toxic than selenite, with Se-Met having an LD_{50} of 6.5 µg/egg compared with selenite with an LD_{50} of 15 µg Se/egg. Sukra *et al*. (1976) also used selenite in a chick embryo study and reported an even lower LD_{50} of 1.5 µg Se/egg when injected via the air cell at d 3, 5, 9 and 15 of incubation.

The goals of this experiment were 1) to determine if using *in ovo* injection at 10d of incubation could enhance se status of the embryo 2) if Se could safely be injected and 3) if Se-Met or Na_2SeO_3 were metabolized differently by the embryo. Therefore the current study was undertaken to investigate the effects of injecting graded level of Se as Se-Met or Na_2SeO_3 on embryo viability and tissue selenium concentrations.

MATERIALS AND METHODS

Preliminary Research Study

Site and Time of Injection

There are different sites in which an avian egg can be injected through the eggshell to gain access to the embryo, including the air cell, the yolk sac, the extra-embryonic coelom, and the amnion. However, previous Se injection studies showed two of these four sites were most commonly used, *i.e*., the yolk sac and air cell. However, since during embryonic development, yolk is the primary source of energy and nutrients including selenium, *i.e*., until the digestive tract is functional, the yolk sac was the most logical route to use for this study. In addition, Ohta *et al*. (2001) showed that the yolk and the extra embryonic coelom were the best injection site for nutrients such as amino acids. Paton *et al*. (2002) demonstrated that the greatest increase in Se concentration in the chick embryo occurred between 10-15 days of incubation. From a practical standpoint, the outline of the yolk at 10 d of incubation is large enough to seen through the shell during candling, in addition, identifying the embryo becomes easier then.

Location of the Yolk

To deliver the nutritive solution into the yolk by injection without the risk of hitting the embryo or any major blood vessel it was necessary to candle each egg to locate the embryo, which appeared as a dark floating silhouette and the head as a dark spot. Usually under the candling light, healthy embryos move in response to the light. However, there are some embryos that tend to be sluggish and take a little bit more time to start moving. The egg was turned about a quarter turn away from the head, to locate the yolk, the target site of injection.

Preliminary Research Study - Verification of Injection Procedure

One of the most critical point in this experiment was to determine that yolk was the site where the solution is being delivered. Therefore to, ascertain this, a food grade blue dye diluted in PBS was injected into the yolk of 85 fertile eggs after 10 days of

incubation. Sterile water and PBS were also included as injection control solutions, using 15 eggs for each. The blunt end of the egg was held under the candler and the position of the embryo as well as the yolk was determined as described above. The outline of the air cell was traced around the shell and a small hole was drilled onto the egg shell. Eggs were injected with 0.5ml food grade dye in PBS, sterile water or PBS alone. Holes were sealed using glue and eggs were then returned to the incubator. At Day 18 of incubation, eggs were candled prior to transfer to the hatcher. At the same time, five eggs from the dye-injected group were sampled for inspection of the correctness of injection site.

Day of Hatch

At day of hatch, baskets containing the chicks were pulled out of the incubator and the numbers of chicks alive and dead were recorded.

Study 1 - In Ovo Injection of Graded Levels of Se

This experiment was conducted at the University of Kentucky Poultry Farm. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC). Fertile white eggs from Hy-Line W-36 hens (Hy-Line International, IA) fed a low-Se diet were used for this study. Fertile eggs were incubated at 37.5°C and a relative humidity of 55 to 60% during d 1-18 and at 36 °C with relative humidity of 60-65% during d 19-21. Two sources of Se, Se-Met and Na_2SeO_3, were used at five injection doses each (0, 2.5, 5, 10 or 20 µg Se/egg). At least 30 eggs were allotted to each injection treatment. Sham-control eggs injected with phosphate buffered saline (PBS) and non-injected eggs were also included. Average egg weight was approximately 50 g.

Preparation of Se Solutions

Sterile phosphate buffered saline (PBS) was prepared with Sigma® PBS tablets (Sigma®, St. Louis, MO) (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) according to manufacturer's instructions and was autoclaved at 121°C for 45 min. Seleno-L-methionine (Sigma®) or Na_2SeO_3 (Sigma®) was dissolved in PBS to make a 200 µg Se/ml stock solution which was subsequently diluted with PBS to make

solutions containing 100, 50 and 25 µg Se/ml. All solutions were filtered using a 0.22 µ acetate/pre filter (MSI®, Westborough,MA).

Se Injection

At d 10 of incubation, eggs were removed from the incubator and candled for viability. Under the candler, the outline of the air cell was traced using a pencil and the location of the embryo marked. After disinfecting the shell with an alcohol swab, a small hole was drilled on the larger end of the egg, above the air cell. Using a 23 gauge, 1 ½" (0.6mm x 40mm) needle, 0.1 ml of the Se solution was injected into the yolks to provide doses of 20, 10, 5, 2.5 and 0 µg Se. The injection site on the shell was sealed with glue and eggs were returned to the incubator.

Tissue Sample Collection

After 20 d of incubation eggs were removed from the incubator and the embryos were euthanized through cervical dislocation for liver, lung and heart tissue collection. For each treatment group, three pooled samples of 10 tissues were collected. Samples were frozen at -20°C until analysis.

Analytical Procedures

Liver, lung and heart samples were analyzed for Se content according to the fluorometric assay following nitric-perchloric acid digestion described by Olson *et al*. (1975) with modifications by Cantor and Tarino (1982).

Statistical Analyses

Results were analyzed using the general linear model for ANOVA procedures, there was a factorial arrangement of treatments using two Se sources and 5 injection doses. Se source x dose treatment means were separated by the test of least significance difference (LSD). Linear regression was performed to analyze the relationship between tissue Se concentration in response to the doses injected (Statistix 9.0). Comparison of slope was performed to test the difference between the two regression lines.

RESULTS AND DISCUSSION

Preliminary Research Study

For the preliminary study, Figure 3.1,A, depicts the location of the embryo in relation to where the yolk was located. At day 18 of incubation, upon opening the five eggs injected with blue dye, it was noted that color of the yolks had a greenish tinge compare with the normal yellow color of uninjected eggs (Figure 3.1,B), probably the combination of yellow yolk and blue food grade dye that was injected. At day of hatch, the newly hatched chicks also possessed bluish tinged-down. Viability is shown in Table 3.1. The dye injected fertile eggs had 91% viability, which wad similar to the values for PBS or water injected fertile eggs with 93%. The preliminary results indicated that the injection technique had minimal effect on embryo viability and mortality attributed to injection trauma was very limited. Therefore, the subsequent research study on *in ovo* injection of Se could be carried out using the procedures of the preliminary trial.

Study 1 – In Ovo Injection of Graded Levels of Se

The effects of graded doses (0, 2.5, 5, 10 and 20 µg/egg) of Se either Se-Met or Na_2SeO_3 injection on embryo viability 10 d post-Se injection is summarized in Table 3.2. Embryo viability ranged from 84 to 97% for eggs injected with graded doses of Se-Met and from 74 to 94% for those injected with Na_2SeO_3. The viability of embryos for PBS-injected and the non-injected eggs was 90 and 100%, respectively. The Se-Met-injected eggs had higher embryo viability (percentage of live embryos of injected eggs) compared with the Na_2SeO_3 group. Upon opening each egg, the presence of any signs of abnormalities, such as inflammation of the neck and head region, as well as beak, leg and toe deformities (Franke *et al*., 1936), were noted and embryo deaths were recorded. For this study, neither the injection of Se-Met nor the Na_2SeO_3 produced any untoward abnormalities in the embryo, in contrast to reports from previous Se injection studies. Our finding may indicate that both Se sources up to 20 µg Se/egg (based on 50-g egg weight) did not affect the development of the embryo enough to produce abnormal growth.

The LD_{50} of Se injected in chicken eggs at 9 d of incubation has been reported as approximately 0.030 ppm when given via the air cell (Sukra et. al., 1976). In contrast, when injected via the yolk sac at 8 d of incubation, investigators found the LD_{50} to be 0.4 ppm (20 µg/egg) (Ridgway and Karnofsky, 1952) and between 0.01 to 0.025 mg/egg (18 µg/egg) when administered at 4 d of incubation (Kury *et al*., 1973). Survival rate in the Ridgway and Karnofsky (1952) study was 10-20% compared with 28% livability (or 72% mortality) in the study by Kury *et al*. (1967). In a study by Palmer *et al*. (1973), the toxicity of Se as Se-Met was similar to that as selenate (LD_{50} = 0.13 µg/ml or 5.46 µg/egg), but greater than that as selenite (LD_{50} = 0.3 µg/ml or 12.6 µg/egg). They found that the ratio of live chicks to total fertile eggs injected decreased from 16/17 to 2/18 when the injected Se concentration increased from 0.1 to 0.4 ppm. However, in the current study, we observed that injecting up to 20 µg Se/egg did not negatively affect embryo viability and, therefore, was not found to be a toxic dose.

The overall effect of Se *in ovo* injection on liver, lung and heart muscle Se concentrations is shown on Table 3.3A. Regardless of Se source, the Se content of the liver was higher than that of the lung and heart muscle. This result is consistent with previously reported studies on Se distribution and retention in the liver (Leeson *et al*., 2008; Pan *et al*., 2007; Payne and Southern, 2005).

Selenium concentrations in the liver linearly increased with graded levels of Se injected for both the Na_2SeO_3 and Se-Met (Figure 3.2). However, the regression coefficient (Table 3.3B) was significantly greater for Na_2SeO_3 than for Se-Met (0.06 vs 0.01).

The effect of Se injections also resulted in linear increases in tissue Se in lung, except resulting concentrations were lower compared with liver. For example, injecting 20 µg Se as Na_2SeO_3 resulted in a Se concentration of 1.517 µg/g in liver but only 0.664 in lung (wet basis) (Figure 3.3). The regression coefficient for the Na_2SeO_3 was significantly higher than that for Se-Met ($P < 0.001$) (0.02 for Na_2SeO_3 vs 0.01 for Se-Met). Figure 3.4 depicts the effect of Se injection on the heart muscle.

Linear increases for tissue Se concentrations were observed in this experiment however Na_2SeO_3 was higher than Se-Met. These result were also demonstrated by

Noguchi *et al*. (1973) wherein at low Se concentration, Na_2SeO_3 was slightly effective in stimulating plasma GSH-Px activity than Se-Met.

Omaye and Tappel (1974) also showed this dose-response relationship in 20-24 d old chicks, that at low Se concentrations, the liver and the heart soluble fractions had slightly higher mean GSH-Px activities than Se-Met. On the other hand, Cary *et al*. (1973) concluded that dietary Se levels given at less than 0.1 ppm in weanling rats showed no difference in tissue Se retention between Na_2SeO_3 and Se-Met. Consequently when dietary supplementation was more than 0.1 ppm, tissue Se retention was higher for Se-Met than Na_2SeO_3. This is parallel to the pig study conducted by Mahan and Parrett (1996), where diets were supplemented with 0.1, 0.3 or 0.5 ppm Se as Na_2SeO_3 or Se-enriched yeast. These authors demonstrated that, at dietary level of 0.1 ppm, serum Se concentration was higher for Na_2SeO_3 than Se-enriched yeast and this resulted in an interaction response between these two variables ($P < 0.01$). On the contrary, Whanger and Butler (1988), rats fed with different levels of Se (0.2, 1.0, 2, or 4 mg/kg), and it revealed that at a level of 0.2 ppm tissue Se concentration did not differ between Se-Met and Na_2SeO_3. However, when Se supplementation was increased, Se-Met tissues expressed higher accumulation of Se-Met than Na_2SeO_3.

In contrast to previously reported Se-injection studies, our study demonstrates for the first time that injecting white shelled fertile eggs after 10 d of incubation via the yolk sac with doses up to 20 µg Se does not adversely affect embryo viability. These unique observations could probably be attributed to the route of injection and the day of incubation when the eggs were injected. Furthermore, liver and lung, but not heart, Se concentrations were found to be higher in response to Na_2SeO_3 than Se-Met from tissues harvested at 20 d of incubation. The effects of the Se-Met and Na_2SeO_3 on liver and lung Se concentrations suggest that these compounds are metabolized differently by the chick embryo.

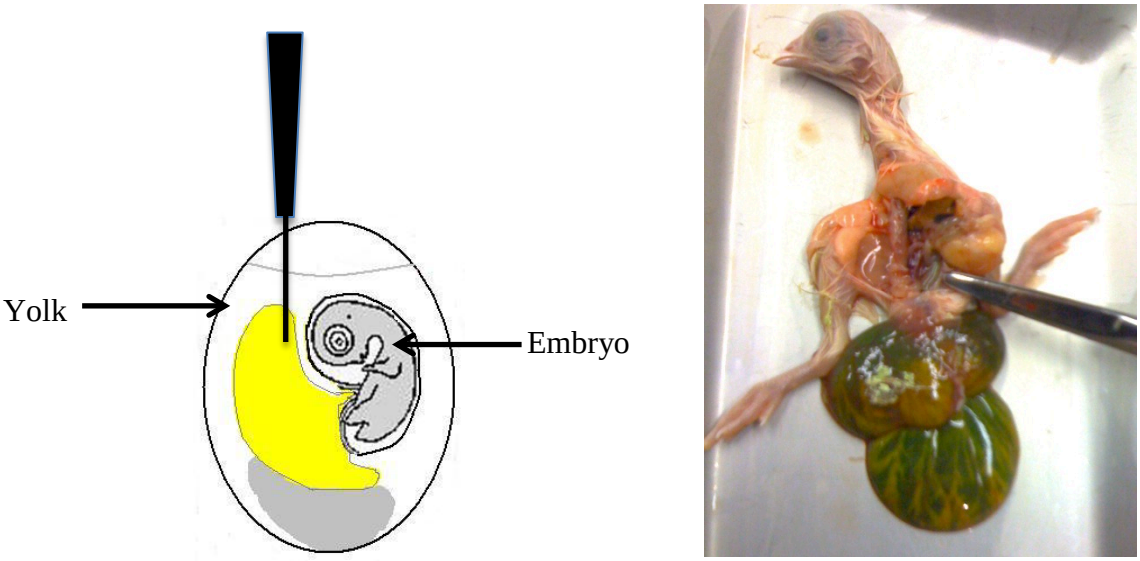


Figure 3.1. Yolk sac injection at d10 of incubation and presence of blue dye
A) Schematic diagram showing injection site in the yolk B) Photograph showing the presence of blue dye in the yolk after Day 18 of incubation.

Table 3.1. Results of preliminary *in ovo* blue dye injection study.

	Number of Live embryos			
Injection Solution	Day 10	Day 18	Day 21	Viability, %**
Blue Dye	85	80*	77	91
Water	15	15	14	93
PBS	15	14	14	93

n= the number of live embryos at Day 10
*5 eggs were sampled at d 18, 4 alive, 1 dead
** Viability = (live embryos at d 21÷ Live embryos at d 10)* 100

Table 3.2. Se injection and embryo viability (%) at 20 days of incubation.*

Dose, µg/egg	Se-Met	Na_2SeO_3
0	93.7	93.7
2.5	96.7	87.0
5.0	93.7	93.7
10	90.0	74.0
20	83.0	87.0

Viability = (live embryos at d 21÷ Live embryos at d 10) 100

Table 3.3A. Effect of Se *in ovo* injection on embryonic tissues at 20 d of incubation.

	Se Source					
	Na_2SeO_3	Se-Met	Na_2SeO_3	Se-Met	Na_2SeO_3	Se-Met
Dose	Liver		Lung		Heart	
0	0.348^{f}	0.358^{ef}	0.202^{d}	0.208^{d}	0.315^{a}	0.300^{a}
2.5	0.453^{def}	0.418^{def}	0.219^{d}	0.268^{cd}	0.252^{b}	0.240^{b}
5	0.516^{d}	0.502^{de}	0.246^{cd}	0.274^{cd}	0.238^{b}	0.200^{c}
10	0.808^{b}	0.524^{d}	0.251^{cd}	0.403^{b}	0.252^{b}	0.240^{b}
20	1.518^{a}	0.664^{c}	0.304^{c}	0.538^{a}	0.315^{a}	0.300^{a}
SEM	0.151	0.036	0.042	0.012	0.014	0.013
	P value					
Source	0.000		0.0003		0.071	
Dose	0.000		0.000		0.000	
Source*Dose	0.000		0.005		0.379	

a-f : $P < 0.05$

Table 3.3B. Comparison of slope for tissue Se concentration (μg/g) of embryos at 20 d of incubation.

Tissue	Se Source	Slope	*P* value
Liver	Na_2SeO_3	0.06	< 0.001
	Se-Met	0.01	
Lung	Na_2SeO_3	0.02	
	Se-Met	0.01	< 0.001
Heart	Na_2SeO_3	0.01	
	Se-Met	0.01	0.772

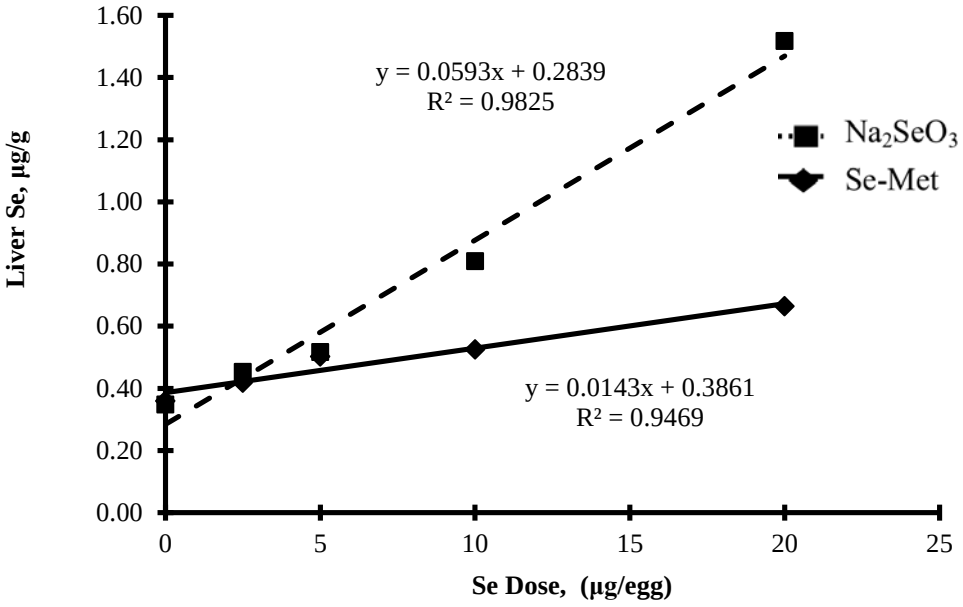


Figure 3.2. Liver Se concentration (wet basis, µg/g)) of 20-day-old embryo injected with Se containing 0, 2.5, 5, 10 or 20 µg Se as Se-Met or Na_2SeO_3.

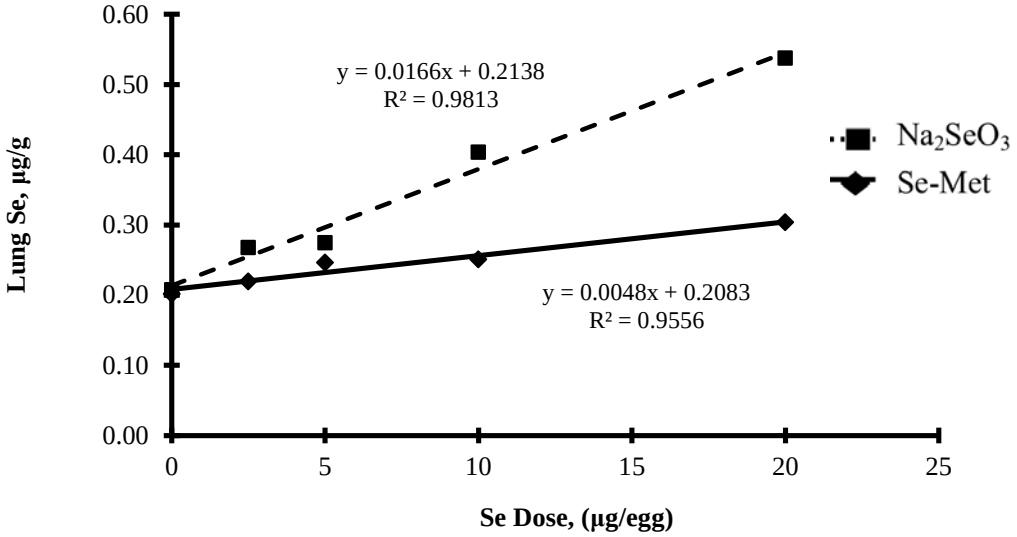


Figure 3.3. Lung Se concentration (wet basis, µg/g) of 20-day-old embryo injected with Se containing 0, 2.5, 5, 10 or 20 µg Se as Se-Met or Na_2SeO_3.

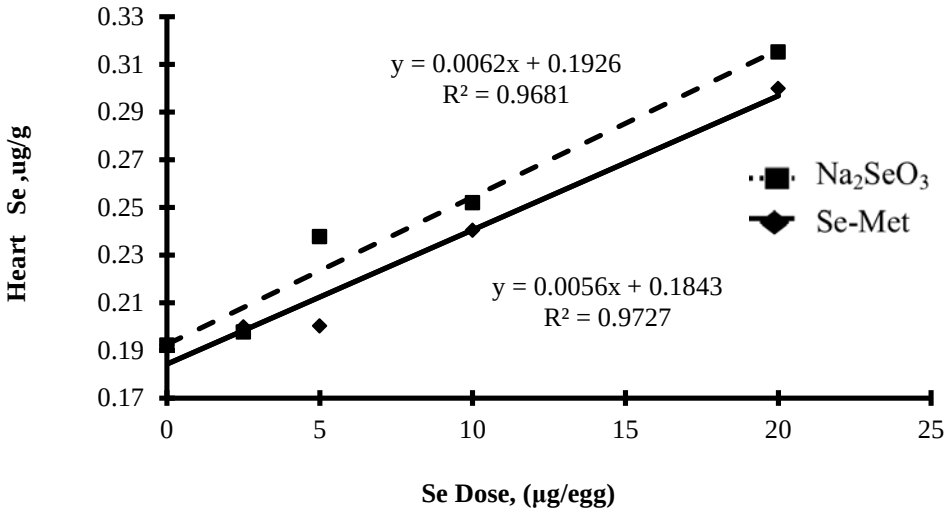


Figure 3.4. Heart Se concentration (wet basis, µg/g) of 20-day-old embryo injected with Se containing 0, 2.5, 5, 10 or 20 µg Se as Se-Met or Na_2SeO_3.

CHAPTER 4: TOXICITY AND TISSUE SELENIUM LEVELS OF CHICKEN EMBRYOS RESULTING FROM *IN OVO* SELENIUM INJECTION

INTRODUCTION

Although Se has been shown in many previous studies to be essential in embryonic development of various poultry species (Thompson and Scott, 1970, Scott *et al*., 1967, Cantor and Scott, 1974, Cantor *et al*., 1982) it has also been reported to be toxic to developing embryos at high levels whether injected *in ovo* (Sukra *et al*, 1976; Palmer *et al*., 1973; Kury *et al*., 1967; Halverson *et al*., 1965; Franke *et al*., 1936; Ridgway and Karnofsky, 1952) or supplemented to hen diets (Heinz and Hoffman, 1996; Ort and Latshaw, 1978)

In a previous experiment, we tested the effect of Se injected into the yolk sac incubating eggs after 10 d of incubation. From that study, we concluded that Se either as Se-Met or Na_2SeO_3 up to 20 µg/egg was not detrimental to the developing embryo. In addition, no physical abnormalities or deformities were noted on embryos as has been reported by previous investigators. Linear increases in total tissue Se concentration were evident as the dose of Se injected increased. Liver and lung Se concentrations were significantly higher for eggs injected with Na_2SeO_3 than Se-Met, but did not differ for Se concentrations in heart tissue.

Based on these results, the present study was conducted to determine the effect of high doses of Se (up to 60 µg/egg) injected in 10-d-old fertile eggs via the yolk sac on livability, and tissue Se concentration.

MATERIALS AND METHODS

Experimental Animals and Treatments

This experiment was conducted at the Alltech-University of Kentucky Research Alliance Poultry Farm. All procedures for this study were conducted under protocols approved by the University of Kentucky Institutional Care and Use Committee (IACUC). Fertile white shelled eggs from Hy-Line W-36 hens (Hy-line® International, IA) were incubated at 37.5°C and at a relative humidity of 55 to 60% during d 1-18 and at 36.9 °C with relative humidity of 60-65% during d 19-20. There were two sources of Se Se-Met and Na_2SeO_3 were used at three Se injection doses (20, 40 and 60 µg Se/egg). At least 25 eggs with four replications were equally allotted to the three Se treatment doses for Se-Met and Na_2SeO_3. Sham controls were also included which were injected with PBS. Average egg weight was approximately 50 g.

Preparation of Se Solutions

Sterile PBS was prepared with PBS tablets (Sigma®, St. Louis, MO) (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) according to manufacturer's instructions and was autoclaved at 121°C for 45 min. Seleno-L-methionine (Sigma®) or Na_2SeO_3 (Sigma®) was dissolved in PBS to make a 100 µg Se solution which was subsequently diluted with PBS to make 60, 40 and 20 µg Se solutions. All solutions were filtered using a 0.22 µ acetate/pre filter (MSI®, Westborough, MA).

Se Injection Procedure

At d 10 of incubation, eggs were removed from the incubator, candled for viability and prepared for Se injection. Under a candler, the outline of the air cell was traced using a pencil and the location of the embryo marked. After disinfecting the egg shell with alcohol swab, a small hole was drilled on the larger end of the egg, above the air cell. Using a disposable tuberculin syringe with a 23 gauge, 1 ½" (0.6 mm x 40 mm) needle, 0.1 ml of the appropriate Se solution was injected into the yolk. The injection site

on the eggshell was sealed with glue and eggs were returned to the incubator with settings according to standard hatchery practices.

Embryo Livability

After 18 d of incubation, eggs were candled and any dead embryos were counted and then discarded. By the end of the study, at 20 d of incubation, all eggs were broken out and visually inspected for signs of physical abnormalities. The number of dead embryos was recorded. The livability (%) was calculated as the number of embryos that were alive on d 20 divided by the number of eggs that were injected on d 10 of incubation multiplied by 100.

Tissue Sample Collection

After 20 d of incubation, embryos were euthanized by cervical dislocation. Two samples of liver, lung, heart and breast muscle were pooled from 5 embryo per source x dose treatment and frozen at -20°C until analysis.

Analytical Procedures

Liver, lung, heart and breast muscle samples were analyzed for Se concentration according to the fluorometric assay of Olson *et al*. (1975) with modifications by Cantor and Tarino (1982). Within each pooled sample, tissues were minced and manually macerated to make each replicate a more homogeneous sample. Macerated tissues were weighed into digestion tubes and digested in nitric and perchloric acids. The Se concentration of liver, lungs, heart and breast muscle was calculated for each Se source and dose by d 20 of incubation.

Statistical Analyses

Results were analyzed by ANOVA. Se source x dose treatment means were separated by the test of least significance difference (LSD). Linear regression analysis was used to compare the relationship between tissue Se concentrations to Se-Met- and Na_2SeO_3 injection doses (Statistix 9.0). Comparison of slope was performed to test the difference between the two regression lines.

RESULTS AND DISCUSSION

The effect of injecting graded levels of Se at 20, 40 and 60 µg/egg either as Se-Met or Na_2SeO_3 did not produce any physical deformities in embryos by d 18 and 20 of incubation, as shown in Table 4.1. At the 60 µg Se dose, % livability was higher ($P < 0.05$) for embryos injected with Se-Met compared with Na_2SeO_3.

The concentration of selenium in tissues is affected by several factors such as the dietary Se concentrations, number of days Se is consumed, the form or source of Se, whether organic or inorganic in nature, and the type of tissue. Animals in utero, or in the case of birds, *in ovo*, Se levels would be highly dependent on the Se level of the maternal diet (Paton *et al*., 2002; Surai, 2000; Cantor and Scott, 1974; Latshaw and Osman, 1974).

Tissue Se concentrations corresponding to each Se source and dose used are presented in Table 4.2. The order of Se concentration in d 20 embryo tissues was liver > lung > heart > breast regardless of Se source. Injecting graded doses of Se as Se-Met or Na_2SeO_3 into the yolk sac of 10-d-old incubating eggs resulted in significant linear increases in the Se concentrations for all of the tissues collected in this study ($P < 0.05$).

The Se levels of liver and lungs at 20 d of incubation are presented in Figures 4.1 and 4.2. In response to either Se-Met and Na_2SeO_3, the pattern of Se concentrations in liver was similar to that in the lung. The liver Se concentrations increased linearly with increasing Se dose using either Se-Met or Na_2SeO_3 (Figure 4.1). However, the liver Se concentrations in response to 60 µg Se as Na_2SeO_3 did not differ from the response to 40 µg Se as Se-Met (2.474 vs 2.049, µg/g). Although both Se sources increased liver Se, the regression coefficient (Table 4.3) for Se-Met was significantly higher than the regression coefficient for Na_2SeO_3 (0.49 *vs.* 0.80). Although, the lung tissue followed the same trend as liver, Se concentrations were about one third of those in the liver. Again at 60 µg, Na_2SeO_3 did not differ from the 40 µg SeMet ($P < 0.05$). These results are similar to those of previous studies (Surai, 1999; Gaál *et al*., 1995) wherein glutathione peroxidase (GSH-Px) expression in the liver of the developing embryo was higher than in the lungs. Selenium is a critical component of GSH-Px, which participates in antioxidant defense in the liver, helping to prevent lipid peroxidation (Rotruck *et al*., 1973). Thus, providing

high levels of Se *in ovo* may be beneficial during embryonic development to prevent possible embryotoxicans present at the time of hatching and onwards. Linear increases in the Se concentration in the heart and breast muscle are shown in Figures 4.3 and 4.4. Se-Met injection resulted in significantly greater accumulation of Se in both tissues compared with Na_2SeO_3. In the breast muscle, regression coefficient for Se-Met was 0.33 compared with 0.06 for Na_2SeO_3 ($P < 0.0001$). In response to Na_2SeO_3, the pattern of Se concentrations in the heart was comparable to that observed in breast muscle, *i.e*., tissue concentrations did not differ in response to the 40 and 60 µg doses (Table 4.3). These tissue levels were similar to those in response to 40 µg Se as Se-Met. Increasing the Se doses from 40 to 60 µg Se as Na_2SeO_3 did not significantly increase the Se concentration in heart nor in breast muscle ($P > 0.05$). The respective Se concentrations obtained at doses of 40 and 60 µg, were 0.508 and 0.513 µg/g for the heart and 0.326 and 0.361 µg/g for breast muscle. Regression coefficients for Na_2SeO_3 and Se-Met in breast tissue were 0.06 and 0.33, respectively (Table 4.3). These findings were similar to those of previous studies, wherein Se concentration in tissue in response to Se-Met continuously increased with increasing Se dose, whereas Se concentrations in tissue in response to Na_2SeO_3 dose eventually leveled off. This result was similar to the effect of dietary Na_2SeO_3 on Se concentrations of whole egg and egg fractions when various levels of dietary Se (0.1-0.3 mg Se/kg feed) were added fed to white laying hen (Paton *et al*., 2002). Egg Se concentrations reached a plateau at dietary levels of 0.2 mg Se/kg feed Se when Na_2SeO_3 was added to the diet but continued to increase when Se yeast was fed.

The observations in the current experiment were similar with those of Cary *et al*, (1973) and Scott and Thompson (1971). The differences observed in Se metabolism and tissue concentration as been explained as follows. Selenium as Na_2SeO_3 in excess of amount equivalent to certain binding sites maybe eliminated from the body whereas seleno-amino acids in excess of the primary binding sites may be incorporated into proteins. It is known that Se-Met replaces methionine during protein synthesis, which results in Se incorporation into tissue proteins. Due to the exponential growth and development of the embryo as it reaches the time of hatching, the rate of protein synthesis is high, thereby allowing for greater Se accumulation as Se-Met.

In ovo injection of Se up to 60 µg/egg did not prove to be toxic to the developing embryo, since this dose did not produce physical abnormalities or increased mortality. Although both Se sources linearly increased the Se levels in embryonic tissue, concentrations in response to Se-Met increased continuously, whereas Se retention in response to Na_2SeO_3 leveled off at the 40 µg dose.

Table 4.1. Embryo livability (%) at day 18 and 20 of incubation.[1]

Treatment	Se Source	Se Dose µg Se/egg	Livability (%)[1,2]	
			Day 18	Day 20
1	PBS	0	96[ab]	95[ab]
2	Na_2SeO_3	20	88[b]	86[b]
3	Na_2SeO_3	40	87[b]	84[b]
4	Na_2SeO_3	60	84[b]	82[b]
5	Se-Met	20	96[ab]	93[ab]
6	Se-Met	40	92[ab]	87[abc]
7	Se-Met	60	100[a]	97[a]
SEM			1.61	1.57

[1]Livability = Live embryos at d18 or 20 ÷ live embryos at d 10)*100
a-c : $P < 0.05$.

Table 4.2 Embryonic tissue Se concentration (wet basis, µg /g) at 20 days of incubation.

Se Source	Dose µg Se/egg	Liver Se µg/g	Lung Se µg/g	Heart Se µg/g	Breast Se µg/g
PBS	0	0.621^{e}	0.261^{f}	0.227^{f}	0.163^{f}
Na_2SeO_3	20	1.426^{d}	0.459^{e}	0.384^{e}	0.247^{e}
Na_2SeO_3	40	2.049^{c}	0.710^{c}	0.508^{d}	0.326^{d}
Na_2SeO_3	60	2.412^{b}	0.855^{b}	0.513^{c}	0.361^{d}
Se-Met	20	1.620^{cd}	0.557^{e}	0.556^{c}	0.484^{c}
Se-Met	40	2.474^{b}	0.866^{b}	0.846^{b}	0.745^{b}
Se-Met	60	3.221^{a}	1.184^{a}	1.184^{b}	1.141^{a}
SEM		0.117	0.023	0.019	0.022
P value					
Source Dose Source*Dose		0.0001 0.0000 0.0000	0.0000 0.0000 0.0000	0.0000 0.0000 0.0000	0.0000 0.0000 0.0000

a-f : $P < 0.05$.

Table 4.3. Comparison of slope for liver, lung, heart and breast muscle Se concentration at 20 days of incubation.

Tissue	Se Source	N	Slope	*P* value
Liver	Na_2SeO_3	12	0.49	0.0032
	Se-Met	12	0.80	
Lung	Na_2SeO_3	12	0.20	
	Se-Met	12	0.30	0.0006
Heart	Na_2SeO_3	12	0.06	0.0000
	Se-Met	12	0.31	
Breast	Na_2SeO_3	11	0.06	$P < 0.001$
	Se-Met	12	0.33	

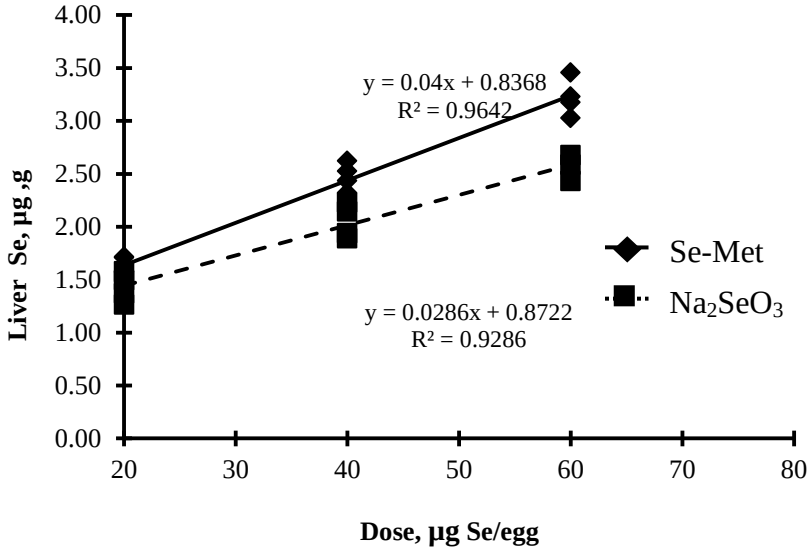


Figure 4.1. Liver Se concentration (wet basis, µg /g) of embryo at day 20 of incubation.

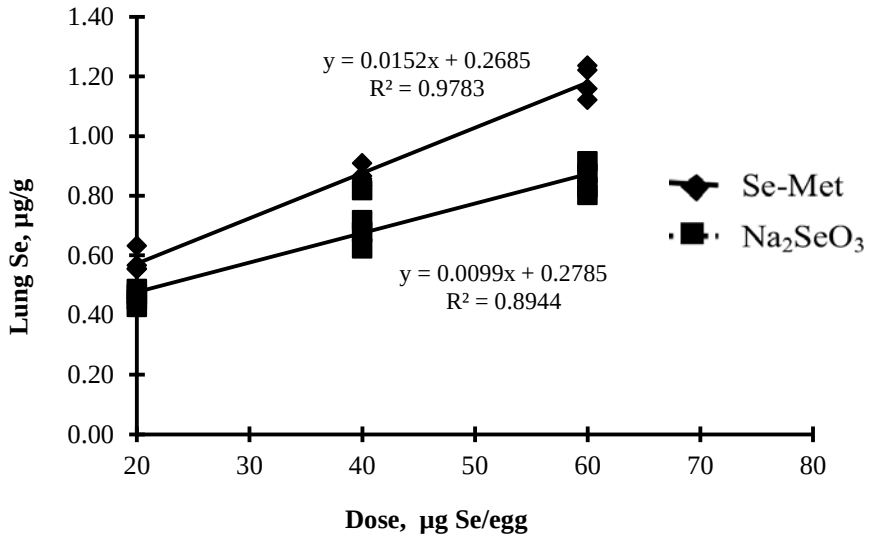


Figure 4.2. Lung Se concentration (wet basis, µg /g) of embryo at day 20 of incubation.

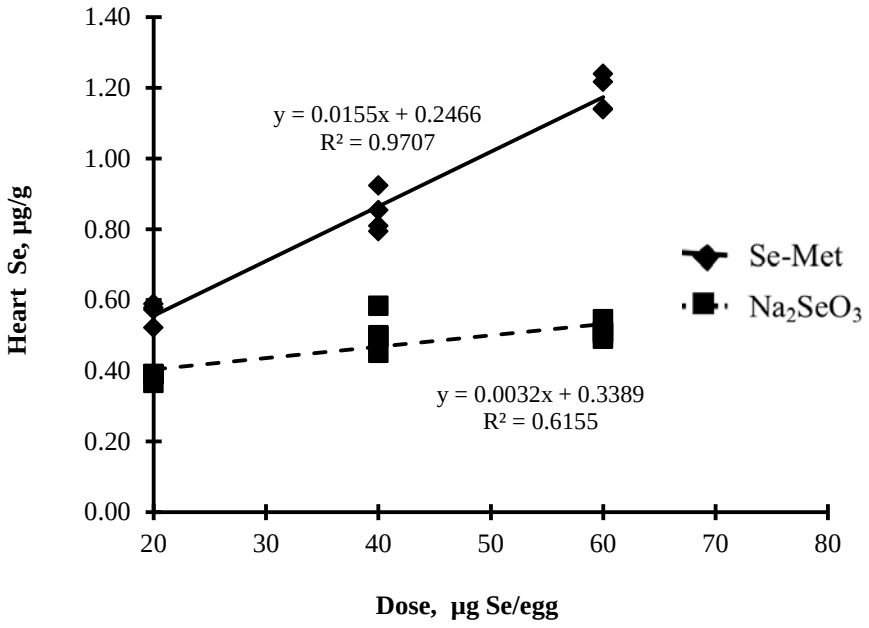


Figure 4.3. Heart Se concentration (wet basis, µg /g) of embryo at day 20 of incubation.

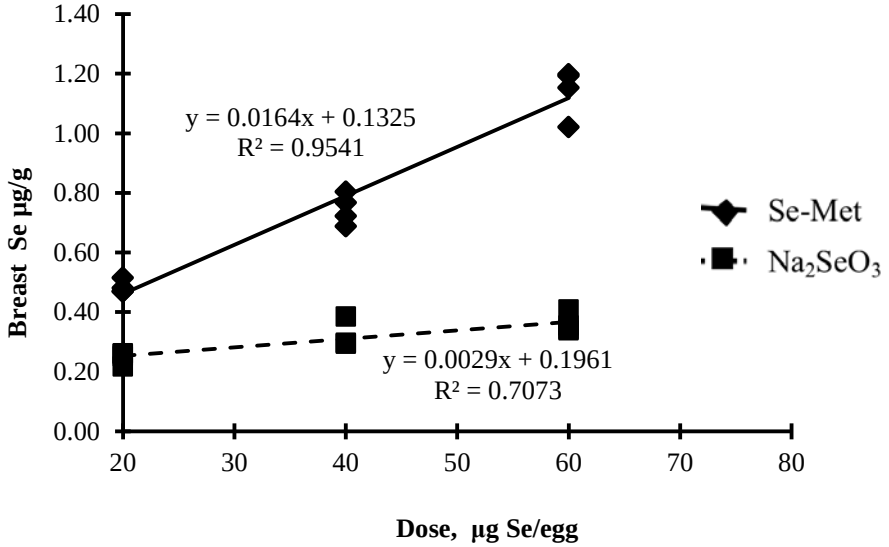


Figure 4.4. Breast Se concentration (wet basis, µg /g) of embryo at day 20 of incubation

CHAPTER 5: EFFECT OF *IN OVO* SE INJECTION OF BROILER BREEDER EGGS AT 10 DAYS OF INCUBATION ON TISSUE SE CONCENTRATION AND EMBRYO VIABILITY

INTRODUCTION

The essentiality of Se in the hen diet has been documented for the growth and development of the chick and turkey embryo (Thompson and Scott, 1970; Scott *et al*. 1967; Cantor and Scott, 1974). Dietary addition of Se has shown that it can be transferred to the yolk, embryo and eventually to the embryonic tissues (Paton *et al*., 2002, Surai, 2000, Pappas *et al*., 2005, 2006). Many nutrient deficiency symptoms seen in young chicks also occur during embryonic development. For example, feeding Se-vitamin E deficient diet to turkey breeder hens resulted in well developed gizzard myopathy in newly hatched poults, indicating that deficiency lesions developed during embryonic growth (Cantor *et al*., 1978).

The amount of Se that can be added to breeder diets is limited by the United States Food and Drug Administration (FDA) to 0.3 mg/kg. Hence, the amount of Se that can be transferred from the hen to the egg is thereby limited. A way to offset this limitation is through early embryo manipulation by injecting Se into the yolk of incubating eggs. This can elevate the levels of Se in the embryo via the yolk. During embryonic development the yolk is the major source of energy and nutrients including Se. It has been reported that yolks from hens fed a low Se diet supplemented with 0.3 ppm Se contained 6-7µg Se (Paton *et al.*, 2002). From these previously mentioned literature Se is deemed essential to the diet not only for the breeders but in the embryo as well. However, earlier investigations on Se injection on the literature resulted in high embryo mortality and embryos that showed physical developmental defects (Sukra *et al*., 1976; Palmer et al., 1973, Kury *et al*., 1967; Halverson *et al*., 1965; Franke *et al*., 1936; Ridgway and Karnofsky, 1952). It was reported that the LD 50 for selenite when injected through the yolk sac ranged from 18-20 µg Se/egg via the yolk sac and 1.5-35 µg Se/egg via the air cell. On the other hand the LD50 for Se-Met was found to be 6.5 µg Se/egg via the air cell. In our laboratory, we have conducted studies on injecting Se into the yolk of 10-day-old fertile white layer eggs. Based on the results of our first study, we have shown that

injecting graded doses *i.e*., 0, 2.5, 5.0 10 and 20 µg Se either as Se-Met or Na_2SeO_3 did not produce any developmental abnormalities and low embryo mortality was observed at d 20 of incubation (Macalintal *et al*., 2010a). Linear increases on tissue Se accumulation resulting from injecting both Na_2SeO_3 and Se-Met were observed. In our second study, we increased the Se injection dose up to 60 µg Se/egg, and it was determined that injecting up to 60 µg Se did not negatively affect embryo viability (Macalintal *et al*., 2010b). Linear increases in tissue Se were also observed as the injection doses increases, similar to the low dose injection study.

These aforementioned studies in our laboratory were conducted in Leghorn eggs, thus, the current study was undertaken to verify these results using broiler eggs. Improved tissue Se levels at hatching may help broilers overcome environmental stresses and disease challenges associated with method of rearing. Therefore, the aim of this study is to investigate the effects of injecting Se up to 40 µg Se into the yolk of 10-day-old incubating broiler eggs on tissue Se concentration and embryo toxicity.

MATERIALS AND METHODS

Experimental Animals and Treatment Groups

This experiment was conducted at the Alltech-University of Kentucky Research Alliance Poultry Farm facilities. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC). Six hundred (600) Cobb® 500 fertile brown eggs obtained from Cobb-Vantress, Monticello, KY were used for this study. Fertile eggs were incubated at 37.5°C and a relative humidity of 55 to 60% during d 1-18 and at 36.9°C with relative humidity of 60-65% during d 19-21. Two sources of Se, Se-Met and Na_2Seo_3 and six Se doses were used (0, 2.5, 5, 10, 20 and 40µg/egg) to inject the eggs. At least thirty (30) eggs were allotted to each injection treatments. Sham control eggs injected with phosphate buffered saline (PBS). All solutions were prepared in sterile PBS solution. Total weight of egg contents was approximately 40g.

Preparation of Se Solutions

Phosphate buffer saline (PBS) was prepared with Sigma® (Sigma, St. Louis, MO) PBS tablets (10mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) according to manufacturer's instructions and was autoclaved at 121°C for 45 min. Se-Met (Sigma®) or Na_2SeO_3 (Sigma®) was dissolved in PBS to make up a 600 µg Se /ml Se solution which was subsequently diluted with PBS to make up 400, 200,100, 50 and 25 µg Se/ml solutions. All solutions were filtered using a 0.22µ acetate/pre filter (MSI®, Westborough, MA).

Se Injection Procedures

At d 10 of incubation, eggs were removed from the incubator and candled for viability. Under the candler, the outline of the air cell was traced using a pencil and the location of the embryo marked. After disinfecting the eggshell with an alcohol swab, a small hole was drilled on the larger end of the egg, above the air cell. Using a disposable tuberculin syringe with a 23 gauge, 1 ½" (0.6mm x 90 mm) needle, 0.1ml of the appropriate Se solution was injected into the yolk. The injection site on the eggshell was sealed with glue and eggs were returned to the incubator with settings according to standard hatchery practices.

Transfer to Hatcher at Day 18

At day 18 of incubation eggs were carefully candled for viability prior to transfer to the hatcher.

Day 20 Tissue Sample Collection

At 20 d of incubation, eggs were removed from the incubator and cracked open for liver, lung, heart and breast muscle tissue collection. Five pooled samples of each tissue were collected per Se treatment. Samples were frozen at -20°C until analysis.

Analytical Procedures

Liver, lung and heart samples were analyzed for Se content according to fluorometric assay by Olson *et al*. (1975) with modifications by Cantor and Tarino (1982). Three pooled samples of 5 tissues were used per treatment group.

Statistical Analyses

Results were analyzed by ANOVA and Se source x dose treatment means were separated by the test of least significance (LSD). Linear regression analysis was used to compare the linear response between the tissue Se concentration and doses injected into the eggs using either Se-Met or Na_2SeO_3 (Statistix 9.0). Comparison of slope was performed to test the difference between the two regression lines.

RESULTS AND DISCUSSION

Injecting 10 d old broiler eggs via the yolk sac with graded levels of Se either as Na_2SeO_3 or Se-Met resulted in low embryo mortality. No abnormalities such as head and neck enlargement nor leg and toe malformations were observed when incubating eggs were injected with doses up to 40 µg Se/egg at 18 and 20 days of incubation. These were comparable with the results from our pervious leghorn eggs studies. These observations were in contrast to earlier reports which suggested that injecting Se into fertile eggs resulted to high mortality and embryos with physical abnormalities such as craniofacial deformities, and limb reduction (Franke *et al*., 1936, Halverson *et al*., 1965; Palmer *et al*, 1973). The probable difference in our results could be due to the following reasons; route of administration, time of injection, quality of solutions used and quality of injection procedures.

The effects of *in ovo* Se injection on livability are shown on Tables 5.1 and 5.2. At d 18 and 20 (Table 5.1), livability was not affected by source nor source x dose interaction but it was significantly affected by dose. At d 18 of incubation, embryo livability for Na_2SeO_3 and Se-Met injected eggs ranged from 98-78 and 100-78% respectively. In Table 5.2, the significant effect on dose at d 18 indicated that eggs

injected above 20 µg Se/egg resulted to lesser embryo survival than eggs injected with less than 20 µg Se/egg. On the other hand, injecting more than 10 µg Se/ egg decreased livability at d 20 of incubation. Livability may be decreased but it was not enough to produce embryonic abnormalities as previously described.

Linear increases for tissue Se concentrations were observed, as a result of injecting graded levels of Se ($P < 0.01$) either as Na_2SeO_3 or Se-Met. However, linear responses behaved differently for the two Se sources for the liver, lung, heart and breast muscle. The order of tissue Se concentration for this investigation was liver > lungs > breast = heart, which is comparable with previous studies (Surai *et al*., 2006, Apsite and Atlavin, 1994, Omaye and Tappel, 1974).

Higher tissue Se accumulation was observed with Se-Met than when Na_2SeO_3 was injected. In addition, the regression coefficients for tissue Se concentrations vs. injected doses for all tissues were significantly greater for Se-Met than Na_2SeO_3. Possible reason could be related to the difference in the rate of mobilization from the site of injection. Although both Se forms are readily absorbed, compared with Na_2SeO_3 more Se-Met was mobilized from the site of injection to the different embryonic tissues. As more Se-Met were transported from the yolk to the different tissues, more Se as Se-Met was retained. Therefore, greater amount of Se can be directly incorporated in tissue protein for protein biosynthesis and storage. This mechanism elevates the levels of Se in the tissues, which helps elevate the amount of Se as Se-Met (Henry and Ammerman, 1995). In contrast, Na_2SeO_3 although is highly absorbed is not well retained in body tissues since its absorption is similar to that of a mineral (Wolfram, 1999).

The observations in the current experiment were similar with those of Cary *et al*, (1973) and Scott and Thompson (1971). The differences observed in Se metabolism and tissue concentration as 1) Se as Na_2SeO_3 in excess of amount equivalent to certain binding sites maybe eliminated from the body whereas, 2) seleno-amino acids in excess of the primary binding sites may be incorporated into proteins. Se-Met incorporation in protein occurs because a chemical similarity exists between Se-Met and methionine which allows the body to use them interchangeably during protein biosynthesis, tRNAMet cannot discriminate between methionine and Se-Met (Schrauzer, 2000). Thus, any Se-Met that is present in excess can be used for protein synthesis such as the skeletal

muscles (Schrauzer, 2000). On the other hand, Na_2SeO_3 that is not absorbed tend to be eliminated by the body. This could explain the difference the higher deposition of Se-Met than Na_2SeO_3.

The influence of Se yolk injection at 10 d of incubation on liver Se is presented in Figure 5.1. Both Se sources linearly increased the liver Se concentration at d 20 of incubation. Increasing the dose likewise increased the Se uptake by the liver. However, the slope for Se-Met (0.082) is significantly higher (P = 0.012) compared with that of Na_2SeO_3 (0.02) (Table 5.4). This means that the rate of Se tissue accumulation is higher for Se-Met than Na_2SeO_3. During incubation, the injected Se was transferred from the yolk to the liver, however, more Se as Se-Met was transported than Na_2SeO_3. This clearly shows that Se concentration in the liver is dependent on Se concentration injected as well as Se source. Surai (1999) and Surai *et al*., (1997), reported that GSH-Px had the highest activity at all phases of the chick development particularly in the liver. These authors have shown that the liver GSH-PX activity rises rapidly during d 10-15 of incubation. This is important because, Paton *et al*. (2002) noted that the greatest increase in the accumulation of Se occurred between d 10-15 of incubation when the average Se concentration was 0.05 µg/g. Thus, an increase in GSH-Px activity would require an additional maternal dietary Se (Hassan 1986; Surai, 2000). Therefore the increase in Se as Se-Met in liver afforded by *in ovo* injection would be beneficial to the embryo considering the previous statements.

Meanwhile, Figure 5.2 depicted the effects of injecting Se on lung tissues. Similar with the liver Se, linear increases in lung Se resulted from injecting Se either as Se-Met or Na_2SeO_3. Again, more Se in the form of Se-Met was being delivered to the embryonic lung tissues via the yolk at day 20 of incubation than Na_2SeO_3. The increase in the slope of Se-Met is significantly higher (0.020 vs 0.070) than the increase for Na_2SeO_3 (P < 0.001), meaning that the rate of tissue uptake is higher for Se-Met (Table 5.4).

The increase in breast muscle Se from embryos receiving Se-Met injection at day 10 of incubation is depicted in Figure 5.3. Tissue Se concentration for the breast muscle is similar to that of heart muscle Se (Figure 5.4). There was a significantly greater rise in

the slope of the lung and breast muscle Se (Table 5.4) resulting from Se-Met injection in comparison with Na_2SeO_3, where $P < 0.001$. Overall, for the tissue Se concentration, the hand, Na_2SeO_3 that is not absorbed tend to be eliminated by the body. This could explain the difference the higher deposition of Se-Met than Na_2SeO_3.

The influence of Se yolk injection at 10 d of incubation on liver Se is presented in Figure 5.1. Both Se sources linearly increased the liver Se concentration at d 20 of incubation. Increasing the dose likewise increased the Se uptake by the liver. However, the slope for Se-Met (0.082) is significantly higher ($P = 0.012$) compared with that of Na_2SeO_3 (0.02) (Table 5.4). This means that the rate of Se tissue accumulation is higher for Se-Met than Na_2SeO_3. During incubation, the injected Se was transferred from the yolk to the liver, however, more Se as Se-Met was transported than Na_2SeO_3. This clearly shows that Se concentration in the liver is dependent on Se concentration injected as well as Se source. Surai (1999) and Surai *et al*., (1997), reported that GSH-Px had the highest activity at all phases of the chick development particularly in the liver. These authors have shown that the liver GSH-PX activity rises rapidly during d 10-15 of incubation. This is important because, Paton *et al*. (2002) noted that the greatest increase in the accumulation of Se occurred between d 10-15 of incubation when the average Se concentration was 0.05 µg/g. Thus, an increase in GSH-Px activity would require an additional maternal dietary Se (Hassan 1986; Surai, 2000). Therefore the increase in Se as Se-Met in liver afforded by *in ovo* injection would be beneficial to the embryo considering the previous statements.

Meanwhile, Figure 5.2 depicted the effects of injecting Se on lung tissues. Similar with the liver Se, linear increases in lung Se resulted from injecting Se either as Se-Met or Na_2SeO_3. Again, more Se in the form of Se-Met was being delivered to the embryonic lung tissues via the yolk at day 20 of incubation than Na_2SeO_3. The increase in the slope of Se-Met is significantly higher (0.020 vs 0.070) than the increase for Na_2SeO_3 ($P < 0.001$), meaning that the rate of tissue uptake is higher for Se-Met (Table 5.4).

The increase in heart Se from embryos receiving Se-Met injection at day 10 of incubation is depicted in Figure 5.3. Tissue Se concentration for the breast muscle is similar to that of heart muscle Se (Figure 5.4). There was a significantly greater rise in

slope of the lung and breast muscle Se (Table 5.4) resulting from Se-Met injection in comparison with Na_2SeO_3, where $P < 0.001$. Overall, for the tissue Se concentration, the highest slope ratios for Se-Met: Na_2SeO_3 were observed with lung (2.9) and heart (3.6) tissues (Table 5.4).

This work demonstrates that injecting Se *in ovo* up to 40µg Se/egg as Se-Met or Na_2SeO_3 does not result in high embryonic mortality and abnormalities. The Se injected into the yolk either as Se-Met or Na_2SeO_3 is readily taken up by the embryonic tissues. It is also clear that Se-Met results in higher Se tissue accumulation compared with Na_2SeO_3 on an equal amount basis. Taken together, the data presented on this work verified the earlier results we reported on our Leghorn eggs studies.

Table 5.1. Embryo livability (%) at day 18 and 20 of incubation.[1,2]

	Day18		Day 20	
Treatment dose	Se Source			
µg Se/egg	Na_2SeO_3	Se-Met	Na_2SeO_3	Se-Met
0	100	93	90	84
2.5	98	98	90	89
5	92	98	88	90
10	96	92	86	80
20	94	78	83	66
40	78	82	71	72

[1]Livability = (viable embryos ÷ fertile eggs set)* 100.
[2]Significant effect: Dose.

Table 5.2. Effect of Se dose on embryo livability (%) at day 18 and 20 of Incubation.[1,2]

Treatment Dose	Livability, %	
µg Se/egg	d 18	d 20
0	97[a]	87[a]
2.5	98[a]	89[a]
5	95[a]	89[a]
10	94[a]	83[ab]
20	86[ab]	71[b]
40	80[b]	71[b]
P value	0.048	0.018
SEM	5.73	8.11

[1]Livability = (viable embryos ÷ fertitle eggs set)* 100.
[2]Significant effect: Dose.
a-c: $P < 0.05$.

Table 5.3. Effect of *in ovo* Se injection on embryonic tissue Se concentration at 20 days of incubation.

Se Source	Dose µg Se/egg	Tissue Se, µg/g			
		Liver	Lung	Heart	Breast
Na_2SeO_3	0	0.979^{ef}	0.237^{g}	0.161^{f}	0.162^{f}
Na_2SeO_3	2.5	0.871^{f}	0.316^{fg}	0.176^{ef}	0.168^{f}
Na_2SeO_3	5	1.044^{ef}	0.334^{ef}	0.187^{ef}	0.175^{f}
Na_2SeO_3	10	1.162^{ef}	0.451^{d}	0.261^{d}	0.241^{de}
Na_2SeO_3	20	1.809^{d}	0.415^{d}	0.236^{de}	0.355^{c}
Na_2SeO_3	40	3.260^{b}	0.569^{c}	0.3591^{c}	0.569^{b}
Se-Met	0	0.893^{ef}	0.265^{fg}	0.194^{ef}	0.181^{ef}
Se-Met	2.5	0.997^{ef}	0.290^{fg}	0.196^{ef}	0.219^{def}
Se-Met	5	1.093^{ef}	0.393^{de}	0.294^{d}	0.274^{d}
Se-Met	10	1.511^{de}	0.420^{d}	0.263^{d}	0.355^{c}
Se-Met	20	2.567^{c}	0.683^{b}	0.533^{b}	0.554^{b}
Se-Met	40	3.992^{a}	1.039^{a}	0.922^{a}	0.869^{a}
Se Source Dose Source*Dose		*P* value 0.024 0.000 0.284	*P* value 0.000 0.000 0.000	*P* value 0.000 0.000 0.000	*P* value 0.000 0.000 0.001

a-f : $P < 0.05$.

Table 5.4. Comparison of slope between Se-Met and Na_2SeO_3 on tissue Se concentration.

Source		Slope	*P* value
Liver	Se-Met	0.081	0.012
	Na_2SeO_3	0.060	
Lung	Se-Met	0.020	<0.001
	Na_2SeO_3	0.007	
Heart	Se-Met	0.018	<0.001
	Na_2SeO_3	0.002	
Breast	Se-Met	0.017	<0.001
	Na_2SeO_3	0.011	

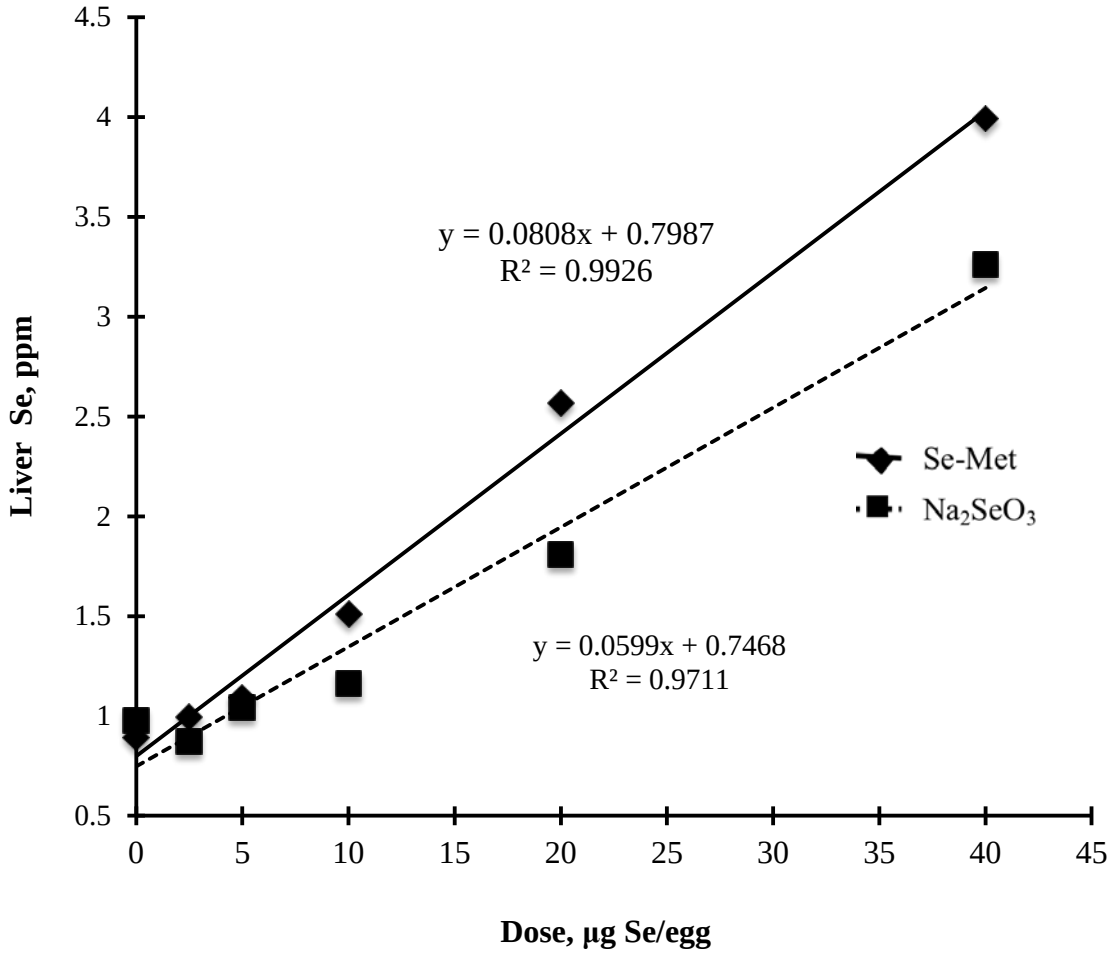


Figure 5.1. Liver Se concentration (wet basis, µg/g) of at Day 20 of incubation.

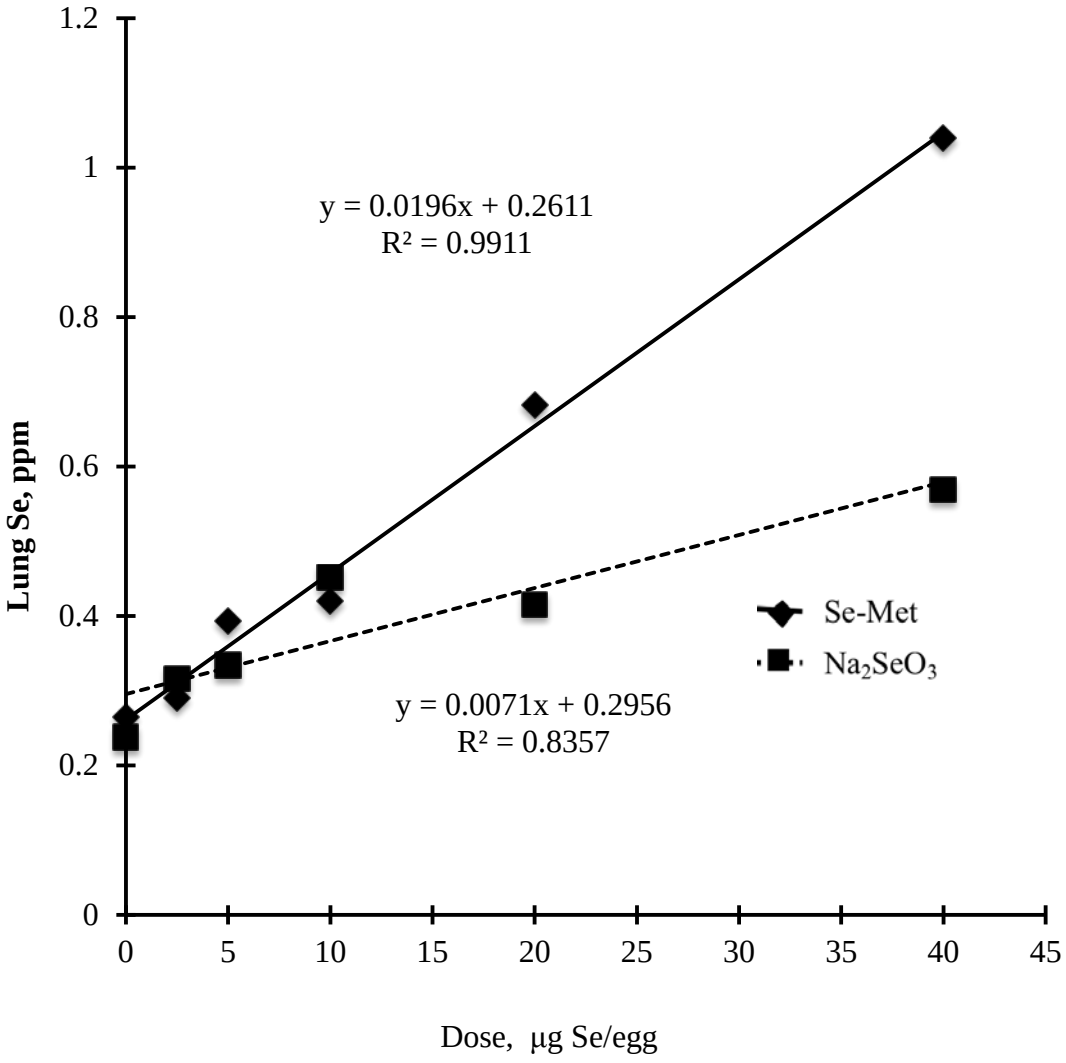


Figure 5.2. Lung Se concentration (wet, basis, µg/g) at Day 20 of incubation.

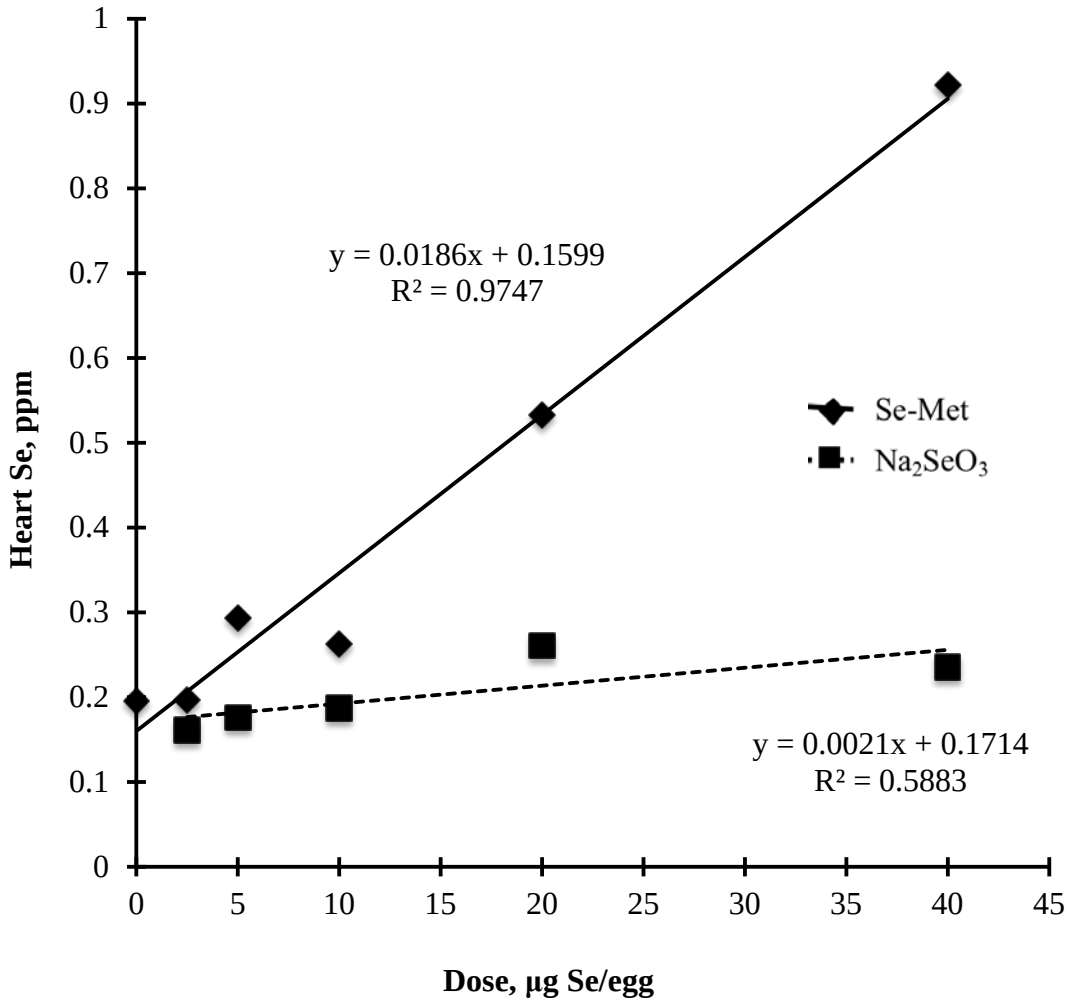


Figure 5.3. Heart Se concentration (wet basis, µg/g) at Day 20 d of incubation.

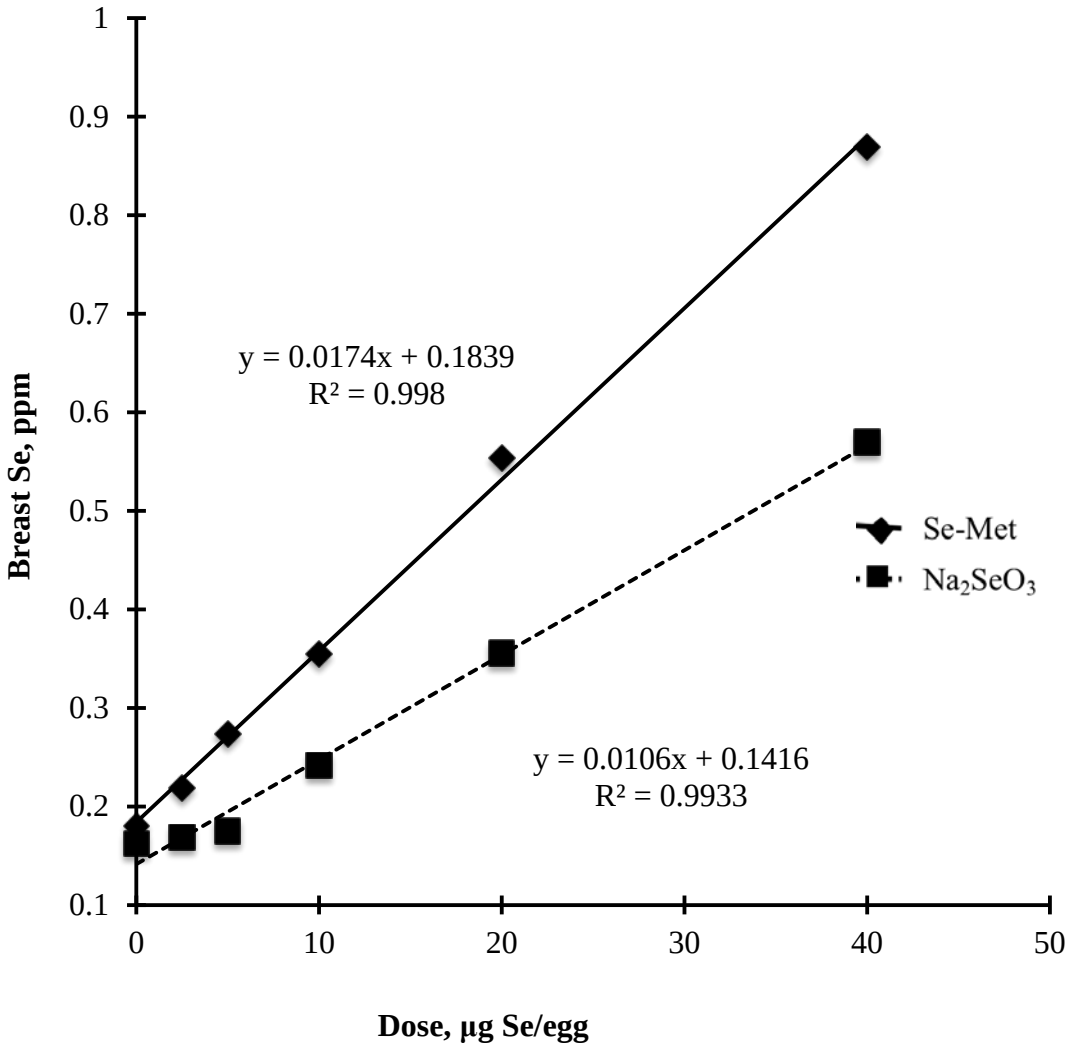


Figure 5.4. Breast muscle Se concentration (wet basis, µg/g) at Day 20 of Incubation.

CHAPTER 6: EFFECT OF *IN OVO* SELENIUM INJECTION OF BROILER BREEDER EGGS ON TISSUE SE CONCENTRATION, LIPID PEROXIDATION, IMMUNE RESPONSE AND POST HATCH DEVELOPMENT

INTRODUCTION

Lipid hydroperoxides when left unchecked can potentially damage the integrity of the cellular membrane and can lead to cell death. These peroxides are the preliminary oxidation products and are further decomposed to secondary products which are aldehydes including malonaldehyde. It has been shown that chick embryo has substantial levels of polyunsaturated fatty acids in the lipid fraction (Speake *et al*., 1998), which are susceptible to oxidation, and therefore needs antioxidant defense system (Surai, 1999). The tissues of the newly hatched chicks contain a variety of natural antioxidants including vitamins A, C, E and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and Se containing glutathione peroxidase (GSH-Px). According to Combs (1981) α-tocopherol and Se are the primary factors in the modulation of oxidant protection. Selenium is a micronutrient that is essential to the nutrition of animals and certain pathologic conditions has been associated with deficiency in diet. However, the effect of Se deficiency in animals was not completely explained until Rotruck *et al*. discovered the biochemical function of Se in 1973. Se is an integral and necessary component of glutathione peroxidase (Glutathione: H_2O_2 oxidoreductase E.C. 1.11.1.9), an enzyme that is responsible in the reduction of hydrogen peroxides (Mills *et al*., 1958) or fatty acid hydroperoxides (O'brien *et al.*, 1969) and responsible for maintenance of sulfhydryls in the reduced state (Srivastava *et al*., 1970). Noguchi *et al*. (1973) demonstrated that day old chicks fed control diets without Se had higher lipid peroxidation than chicks fed a Se supplemented diet. The addition of Se and vitamin E in the chick diet also inhibited the development of exudative diathesis.

Se-dependent-GSH-Px is found in the different cellular compartments (*e.g.*, cytosol, extracellular space, cell membranes). Therefore, the antioxidant properties of selenium are present throughout the cell as well, participating in immune signaling. Se is essential for optimum activity of the immune system (Arthur *et al.*, 2003). Deficiency in dietary Se impacts the competence of both the innate non-adaptive and the acquired adaptive immune system (Spallholz, 1990; Turner and Finch, 1991; Kiremidjian-Schumacher and Roy, 1998; Brown and Arthur, 2001).

Biswas *et al.* (2006) reported that dietary Na_2SeO_3 at 0.5 and 1 ppm increased antibody response against inoculated (sheep red blood cell) SRBC and mitogen response to phytohemagglutinin (PHA) measured as foot web index at 4 weeks compared with the basal diet (0.2 ppm Se) in Japanese quail; Se treatments had no effect on performance. In contrast, Gowdy and Edens (2003) reported that broilers fed organic selenium showed less T-cell-mediated wing web reaction to PHA compared with Na_2SeO_3-fed birds. In a related study, supplementation of Na_2SeO_3 in broiler produced no inhibitory effects on antibody production; however, all antibody titers for Se-supplemented broilers were significantly higher compared with the broilers fed a no Se-supplementation (Gowdy, 2004). However, it was demonstrated by several investigators that addition of higher levels of Se improved the immune functions in young chicks (Biswas *et al.*, 2006; Panda and Rao, 1994; Marsh *et al.*, 1981). Larsen et al (1997) demonstrated that the addition of Se in diet enhanced the immune response of broiler against antigen challenge and reduced the effects of cold stress, 4.9 vs 2.4 $\log_2$ titers. The possible mechanism for the depressed immune function could be related to the effect of inadequeate Se and or vitamin E in the diets resulting to the primary lymphoid organ denegenaration which consequently led to the depletion of lymphocytes (Marsh, 1986, Peng *et al.*, 2011a,b).

The present study was undertaken to evaluate the effects of *in ovo* selenium injection on broiler breeder eggs on tissue Se concentration, lipid peroxidation, immune response and post hatch development.

MATERIALS AND METHODS

Experimental Animals and Treatment Groups

This experiment was conducted at the Alltech-University of Kentucky Research Alliance Poultry Farm facilities at the Coldstream Experimental Unit in Kentucky. All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC). Six hundred (600) Cobb 500 fertile brown eggs obtained from Cobb-Vantress, Monticello, KY were used for this study. Fertile eggs were incubated at 37.5°C and a relative humidity of 55 to 60%. Two sources of Se, 1) Se-Met 2) Na_2SeO_3 and six Se doses were used (0, 2.5, 5, 10, 20 and 40µg/egg) to inject eggs. Two groups of 25 eggs were allotted to each injection treatments. Sham control eggs injected with phosphate buffered saline (PBS). All solutions were prepared in sterile PBS solution. Total weight of egg contents was approximately 40g.

Preparation of Se Solutions

Phosphate buffered saline (PBS) was prepared with Sigma PBS (10mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) tablets according to manufacturer's instructions and was autoclaved at 121°C for 45 min. Seleno-L-methionine (Sigma) or Na_2SeO_3 (Sigma®) was dissolved in PBS to make up a 600 ppm Se solution which was subsequently diluted with PBS to make up 400, 200,100, 50 and 25 ppm solutions. All solutions were filtered using a 0.22µ acetate/pre filter (MSI®).

Se Injection Procedure

At day-10 of incubation, eggs were removed from the incubator and candled for viability. Under the candler, the outline of the air cell was traced using a pencil and the location of the embryo marked. After disinfecting the eggshell with an alcohol swab, a small hole was drilled on the larger end of the egg, above the air cell. Using a 23 gauge, 1 ½" needle, 0.1ml of respective Se solution was injected into the yolk. The injection site on the eggshell was sealed with glue and eggs were placed back into the incubator with settings according to standard hatchery practices.

Transfer to Hatcher at Day 18

At day 18 of incubation eggs were carefully candled for viability prior to transfer to the hatcher.

Day 20 Tissue Sample Collection

At 20 days of incubation, eggs were pulled out of the incubator and cracked open for liver, lung, heart and breast muscle tissue collection. For each treatment groups, 5 pooled samples of each tissue were collected according to each Se level injected. Samples were frozen at -20°C until analysis.

Analytical Procedures

Liver, lung and heart samples were analyzed for selenium content according to fluorometric assay by Olson *et al.* (1975) with modifications by Cantor and Tarino (1982). Three pooled samples of 5 tissues were used per treatment group.

Birds and Diets

At day of hatch, chicks were pulled out of the hatcher for pen placement. Two hundred and eighty eight Cobb® 500 broiler chicks were placed, allotting six birds per cage in a mesh wire-floored standard pullet starter cages (61 cm x 51 cm x 36 cm) in an environmentally controlled room. The temperature in the room was set at 31°C for the first week and adjusted to 27° for the remainder of the study. Continuous light was provided for 22h/day. Each cage was equipped with a one feeder, which is removable for weighing purposes and two adjustable nipple drinkers. Feed and water were supplied *ad libitum*. This experiment used a randomized complete block design. Blocks were based on cage locations within the room. Each experimental unit was composed of one cage per six birds. Four replicate cages were assigned to each of the 12 injection doses used. Treatment doses were randomly distributed to cages within each of the four blocks.

All birds were fed a low-Se corn-soybean meal diet with no Se or vitamin E supplementation. The ingredient composition and the calculated nutrient analysis of the basal diet are given in Table 6.1. This experiment was conducted up to 21 days. Birds

and feed was weighed initially and then on a weekly basis. At the end of each week, 2 birds per cage were euthanized by argon gas asphyxiation followed by cervical dislocation. Liver, lung, heart and breast muscle were collected weekly and stored at -20C until analysis.

Table 6.1. Ingredient and nutrient composition of diet.

Ingredients	% in diet*
Corn	56.25
Soybean meal, 48.5% protein	36.00
Limestone, feed-grade, 38% Ca	1.33
Dicalcium phosphate, 22% Ca, 18.5% P	1.75
Iodized salt	0.45
DL-Methionine	0.19
Vitamin Mix	0.25
Mineral Mix (No Se added)	0.25
Corn or vegetable oil	3.53
Total	100.00
Calculated nutrient composition	
Metabolizable energy, kcal/lb	3087
Protein	22.00
Calcium	1.00
Phosphorus, available	0.45
Methionine	0.51
Methionine + Cystine	0.90
Lysine	1.15
Sodium	0.20

*****unless other unit given

Laboratory Procedures

Lipid Peroxidation of Whole Cell Homogenates Using Ascorbic Acid Stimulation[1]

Tissue Sample Preparation

Two (2g) of tissue sample (liver, lung, heart, breast muscle) was homogenize in 20ml 0.174M KCl in 0.025M Tris-HCl buffer (buffer pH7.4) using a homogenizer. The homogenate was centrifuged at 400 X g for 10 mins and the precipitate was discarded. The supernatant were transferred into new 20ml conical tube.

Lipid Peroxidation Stimulation Using Ascorbic Acid

To the 0.5ml of the whole cell fraction was added with 1ml of 1.5mM ascorbic acid solution in Tris-KCl buffer + 1.5ml Tris-KCl buffer and incubated for 60 minutes at 37°C. The reaction was stopped by adding 1ml of 20% TCA. For the control samples, 1 ml of 20% TCA was added before incubation.

Thiobarbituric Acid (TBA) Lipid Peroxide Formation Test

Samples were filtered using a #541 filter paper (Whatman®, Fisher Scientific, USA). Two (2) ml of filtrate was transferred in clean fresh tubes to which 1 ml of 0.67% TBA was added. Samples were then boiled (100°C) for 35 mins and cool completely in water for 5 minutes. Absorbance was read at 530 mu using a spectrophotometer (Shimadzu® Columbia, MD)

Antibody Production by Sheep Washed Red Blood Cells (SRBC)

Sheep washed red blood cells (SRBC), 100% suspension (Lampire Biological laboratories, Pipersville, PA 18947) was used to stimulate an antibody response. Using two replicates per treatment, all birds from each pen were injected intravenously via the jugular vein with SRBC (1mL/chick of a 7% suspension in sterile phosphate buffered

[1] Modified from Noguchi T, A.H. Cantor, M.L. Scott. (1973).

saline solution) at 3 days of age. Baseline titers were determined by collecting blood samples before SRBC injection. Blood samples were drawn via jugular vein at 7 and 13 days post primary SRBC injection. At 13d post primary injection, birds were given a booster injection (secondary) of SRBC (1ml, 7% SRBC) and blood samples were collected 4 days later to quantify anti-SRBC antibody titers. Sera were extracted and later were stored (-10°C) until analysis. Serum samples were heat inactivated at 56°C for 30 minutes. Samples were analyzed for total, 2-mercaptoethanol-sensitive (ME-S) IgM and mercaptoethanol-resistant (ME-R) IgG. Antibody titers were expressed as the $\log_2$ of the reciprocal of the highest dilution showing 100% visible agglutination.

Statistical Analyses

Results were analyzed by ANOVA and Se source x dose treatment means were separated by the test of least significance (LSD). Linear regression analysis was used to compare the linear responses between the tissue Se concentration and doses of Se-Met and Na_2SeO_3 injected into the eggs (Statistix 9.0).

RESULTS AND DISCUSSION

Embryo livability at d 18 and 20 is shown on Table 6.2, while Table 6.3A showed livability at day of hatch. *In ovo* Se injection did not show any differences between treatments. However, a significant higher source effect was seen for Se-Met than Na_2SeO_3 (Table 6.3B) at day of hatch. In comparison with the previous embryo Se injection conducted in our laboratory (Macalintal *et al.*, 2011b), embryo livability presented in the current study is lower. The difference observed in embryo livability with our previous broiler egg Se injection can be related to the age of the broiler breeder hens. In the current study, eggs were obtained from younger broiler flocks compared to the previous study. This is in agreement with the earlier findings on hatchability with hen age (Hulet *et al.*, 2007; Bruzual *et al.*, 2000; Noble, 1986; Vick *et al.*, 1993; Fasenko *et al.*, 1992; O'Sullivan *et al.*, 1991; Mauldin, 1989; Kirk *et al.*, 1980). It has been reported that eggs from pullets at point of lay had lower hatchability than those eggs laid later in the

cycle (Shanawany, 1984; Mauldin, 1989). Noble (1986) noted a higher embryo mortality from younger flocks due to problems in yolk lipid assimilation and mobilization compared to eggs from older flock. Thus, the lower livability at d 18 and 20 reported here compared to our previous results, can be explained by the age of breeder hens where the eggs were sourced and not due to Se injection *per se*.

Unlike other Se injection studies in the literature (Sukra et al, 1976; Palmer *et al.*, 1973; Kury *et al.*, 1967; Halverson *et al.*, 1965; Franke *et al.*, 1936; Ridgway and Karnofsky, 1952), our experiment did not produce chicks that manifested the head, neck or appendage abnormalities. This finding is in agreement with our previous studies conducted in our laboratory utilizing white-shelled eggs. Thus, we cannot directly correlate Se injection with embryo lethality and abnormality since exposure to higher Se dose in our experiment did not result in such observations. The difference in the findings can be attributed to the quality of solutions used, day and route of injection as well as the quality of injection procedure. For example, Halverson *et al.* (1965) used non-sterile technique in their egg injection procedure except when eggshell was swabbed with 1:1000 solution of tincture of merthiolate prior to injection. In addition, chorioallantoic or air cell injection decreased hatchability to 0 when eggs were injected with amino acids at d 7 or 13% when injected at d 0 (Ohta and Kidd, 2001; Ohta *et al.*, 1999). Thus, the high embryo mortality observed in the Se injection studies in the literature using air cell route cannot be directly attributed to Se, if the above data were to be considered. In a study conducted by Tarantal *et al.* (1991), using long–tailed macaques, these researchers concluded that long-tailed macaque that were orally dosed with Se-Met at maternally toxic doses (up to 300 ug/kg-day) had progeny that did not show any terata. Apart from being different from the previous injection Se studies, our results are in contrast with the Se teratogenic effect from environmental Se exposure (Hoffman *et al.*, 1988). In 1947, Rosenfeld, associated the abnormalities found in neonatal lambs from ewes that grazed in seleniferous areas to congenital microphthalmia. Hale (1937) associated similar defects found in offspring of livestock to vitamin A deficient forage. Ferm and co- workers (1990) concluded that in hamsters, only at doses of overt maternal poisoning *i.e.*, >LD50 and nutritional deprivation can lead to Se-Met induced embryonic toxicity. “Since the physiological basis of selenosis has not been defined and biochemical indicators have not

been identified, it is difficult to distinguish levels of Se that are safe and beneficial to those that are potentially harmful to health " (Ralston *et al.*, 2008). For example, in aquatic birds, several researches have shown that elevated Se exposure can cause reduce reproductive success however; the threshold at which this negative effect is manifested is widely disputed (Adams et al, 2003; Fairbrothers *et al.*, 2000; Ohlendorf *et al.*, 1988, 1989; Heinz *et al.*, 1989; Williams *et al.*, 1989).

Body weights recorded at d 1, 7, 14 and 21 were not affected by the Se injection (Table 6.4). These results are comparable to earlier studies in which supplementation of Se has no significant effect on body weight gain or feed intake (Payne and Southern, 2005b; Utterback *et al.*, 2005; Paton *et al.*, 2002, Cantor *et al.*, 2000).

Tissue Se concentrations for liver, lung, heart and breast muscle at d 7 is presented in Tables 6.5-6.6B. Tissue Se concentration for lung, heart and breast muscle were significantly higher when Se-Met was injected *in ovo* compare with Na_2SeO_3, however there was no difference in the liver Se. Tables 6.7 and 6.8 showed the Se concentration of the chicks at d 14 and 21 respectively. Only the lung Se showed significant difference for both Se source (Table 6.9A) and dose (Table 6.9B), in which Se-Met was higher $P = 0.0001$ (d 14) and $P = 0.0342$ (d 21). Egg formation and embryonic development in oviparous animals like chickens occur outside of the maternal reproductive tract. Oxygen exchange occurs via the air cell which is in direct contact with the outside environment. Thus, chick embryos are constantly challenged by the effects of the outside conditions and may experience greater pulmonary oxygen tension than embryos developing *in utero*. GSH-Px activity, an Se dependent enzyme, in the lung decreases by 27% between 15 and 19 d of incubation and increased by the same amount by d 1 after hatching (Surai, 1999). In addition, Starrs (2001) reported that chick embryos subjected to low oxygen content (hypoxic environment) in the incubator (17.7 vs 20.7%) showed GSH-Px activity that was constant from day 14 to pip, and then increased significantly from pip to hatch. At d 19, respiration switches to pulmonary respiration. Therefore, an increase in GSH-Px activity could be beneficial to the embryo. In the current study, the lung Se concentration was maintained up to 21 days ($P < 0.05$). Thus,

the significant concentration of Se in the lung tissue is important if the above information were to be considered.

Regression analysis for Se tissue concentrations at 7,14 and 21 d of age demonstrated that greater significant rise in slope was observed only at d 7 for the heart muscle. This was not seen in other tissues.

Results for the measurement of the TBARS formation in all tissues collected at d 20 of incubation is presented in Table 6.10. Lipid peroxides formed in tissue homogenates, mitochondrial or microsomal fractions after incubation as measured by TBARS assay has been has been in used for several years (Raharjo *et al.*, 1993; Willis, 1969). Presence of lipid peroxides in cellular membranes had been linked to cellular damages, loss of cellular elasticity and decreased membrane fluidity (Padmaja *et al.*, 1997; Noguchi *et al.*, 1973). For the current study, lipid peroxidation upon incubation with ascorbic acid observed in the lung and the heart was significantly lower when Se source was Se-Met than Na_2SeO_3, whereas the reverse was true for the breast muscle (Table 6.11A). According to Willis (1969), addition of ascorbate in tissue homogenate stimulates the formation of peroxides and is inhibited by glutathione. There was no difference seen in the liver except for the dose effect. Tissue homogenate from embryos that received the 0 μg Se from both Se source had the highest concentration of TBARS (Table 6.11B). Therefore embryos that received 0 μg Se had little natural protection against lipid oxidation compared to the Se-injected eggs. The oxidation products accumulated in tissues are metabolized by the natural antioxidants, such as vitamin A, C and E and the metabolic enzymes such as SOD, CAT and GSH-Px. (Surai, 2000). These compounds prevent the cellular insult than can damage the cellular membrane. Noguchi *et al.* (1973) had previously reported that the mitochondria and microsomal fractions of day old chicks hatched from hens fed a low Se-vitamin E diet showed higher TBARS values compared to supplemented chicks. Protection against lipid peroxidation was afforded with selenium and vitamin E supplementation. In addition, complete prevention of exudative diathesis in these chicks was observed after 12 days of supplementation. According to Padmaja and Prasad (1997) a dose dependent relationship exists between the reduction of TBARS and Se concentration. They reported that the oxidation in the liver and brain homogenate of 14 d old chick embryos decreased as the concentration of

Se injected increased from 0, 12.5 (1.41 mg/g) to 37.5 (4.23 mg/g) μmoles Se/kg egg. In the same study, there was a significant increase in the activity of GSH-Px in the Se treated embryo. GSH-Px works by reducing lipid peroxide to lipid alcohols via the glutathione peroxidase. Therefore dietary addition of Se elevates GSH-Px activity that tends to decrease lipid peroxidation. Omaye and Tappel (1974) also reported that chicks fed a Se enriched diet had increased GSH-Px activity compared Se-deficient fed chicks. Moreover, there is a linear relationship between GSH-Px activity and Se supplementation.

Selenium has been associated not only in the protection of cellular membrane against oxidative stress generated either endogenously or through the metabolic by-products of free radicals, but also with its profound effects on immune response (Levkut *et al.*, 2009; Biswas *et al.*, 2006; Singh *et al.*, 2006; Gowdy and Edens, 2003; Leng *et al.*, 2003; Brown and Arthur, 2001; Swain *et al.*, 2000; Raza *et al.*, 1997; Marsh *et al.*, 1986; Spallholz, 1990; Turner and Finch, 1991; Kiremidjian-Schumacher and Roy, 1998; Colnago *et al.*, 1984; Spallholz *et al.* 1973). The effects of Se in ovo injection of chicks at 3 d of age against SRBC antigen response are shown on Table 6.12. Antibody titers were measured at 0,7,14 d post primary and 4 d post-secondary SRBC injection. Day 0 represents the baseline titer, taken before injecting birds with SRBC. Sera were measured for presence of maternal or none specific antibody titers. For this experiment, d 0 resulted in no hemagglutination of SRBC, thus the presence of maternal or none specific antibody that can interfere with the testing was not detected. SRBC assay measures the antibody response to immunization with SRBC antigens, integrating the function of B lymphocytes, helper T lymphocytes and macrophages (Grasman, 2009). Hemagglutination occurs when the target antigens on the surface of the red blood cells clump together or agglutinate caused by the antibodies directed against them (Janeway *et al.*, 2001). The highest serum dilution at which the concentration of antibody is sufficient enough to agglutinate the SRBC is expressed as the titer. In the case of this experiment, IgMs are presumed to be the antibodies sensitive to the denaturation of 2-mercaptoethanol while IgGs are resistant to it (Yamamoto and Glick, 1982). Antibody titers were not significantly different at 7 and 13 d post primary and 4 d post-secondary SRBC injection (Tables 6.13A-6.13C). However, there was a trend observed at d 7 for all Se-injected birds, the IgM and total antibody titers were numerically higher than the control. The Se-injected chicks had the

highest expression of IgM at 4 d post secondary SRBC injection. This is somehow similar to the result obtained by Larsen *et al.* (1997), where broilers fed graded doses (0.2-0.8 ppm) of Se did not show any in differences in antibody titer among these different doses. However, broilers fed a no Se or control diet expressed significantly lower response than the Se-fed broilers. In our case, the 3 d-old chicks that received 0 μg Se tended to have a numerically lower titer than the Se-injected birds. Despite showing no significant difference between the treatments, it was apparent that the Se injected incubating eggs had a tendency to elicit higher antibody response.

The results of data presented here indicate that *in ovo* Se-Met injection, compared with Na_2SeO_3, resulted in higher hatchability, reduced lipid peroxidation in the lung and heart muscle and higher Se concentrations in heart and breast muscle through 7 days and lung though 21 days of growth.

Table 6.2. Embryo livability (%) at day 18 and 20 of incubation.[1]

Dose µg Se/egg	Day18 Source Na_2SeO_3	Day18 Source Se-Met	Day 20 Na_2SeO_3	Day 20 Se-Met
0	80	83	77	67
2.5	77	77	75	75
5	83	80	80	80
10	75	92	72	86
20	75	87	65	82
40	58	87	52	80
SEM	3.27		3.06	
	----------*P* values----------			
Source	0.0769		0.0927	
Dose	0.7393		0.4520	
Source x dose	0.3918		0.2572	

[1]Livability = % viable embryos / fertile eggs set*100.

Table 6.3A. Embryo livability (%) at day of hatch. [1,2]

Dose, µg Se/egg	Na_2SeO_3	Se-Met
0	72	64
2.5	60	60
5	62	66
10	56	70
20	54	62
40	28	72
SEM	2.65	

[1]Livability = % viable embryos / fertile eggs set.
[2]Significant effect: Source ($P < 0.05$).

Table 6.3B. Effect of Se source on hatchability of fertile eggs (H/F).

Se Source	H/F (%)
Se-Met	66[a]
Na_2SeO_3	55[b]
P value	0.02

a-b : $P < 0.05$.

Table 6.4. Effect of Se injection at 10 days of age on body weights of broiler chicks at 1,7,14 and 21 days of age.

Se Source	Dose μg Se/egg	Age in days			
		1	7	14	21
Na_2SeO_3	0	33	117	341	719
Na_2SeO_3	2.5	34	117	334	736
Na_2SeO_3	5	33	118	311	703
Na_2SeO_3	10	34	119	346	739
Na_2SeO_3	20	33	116	354	742
Na_2SeO_3	40	35	118	360	726
Se-Met	0	33	122	346	702
Se-Met	2.5	33	120	342	719
Se-Met	5	33	118	335	726
Se-Met	10	34	119	345	738
Se-Met	20	34	110	334	725
Se-Met	40	33	123	361	779
SEM *P* value		0.542 > 0.05	4.80 > 0.05	15.75 > 0.05	26.02 > 0.05

Table 6.5. Tissue Se concentration (wet basis, μg/g) of broiler chicks at 7 days of age injected with Se at 10 days of incubation.

Se Source	Dose μg Se/egg	Liver Se μg/g	Lung Se μg/g	Heart Se μg/g	Breast Se, μg/g
Na_2SeO_3	0	0.210	0.126^{cd}	0.139^{c}	0.137^{cde}
Na_2SeO_3	2.5	0.269	0.127^{bcd}	0.150^{bc}	0.132^{de}
Na_2SeO_3	5	0.306	0.131^{abc}	0.157^{bc}	0.146^{bcde}
Na_2SeO_3	10	0.282	0.125^{cd}	0.160^{bc}	0.168^{ab}
Na_2SeO_3	20	0.323	0.124^{cd}	0.174^{b}	0.129^{de}
Na_2SeO_3	40	0.372	0.115^{d}	0.171^{bc}	0.121^{e}
Se-Met	0	0.210	0.126^{cd}	0.139^{c}	0.137^{cde}
Se-Met	2.5	0.300	0.142^{a}	0.172^{bc}	0.154^{bcde}
Se-Met	5	0.309	0.131^{abc}	0.175^{b}	0.140^{bcde}
Se-Met	10	0.303	0.130^{abc}	0.172^{bc}	0.156^{abcd}
Se-Met	20	0.329	0.140^{ab}	0.225^{a}	0.184^{a}
Se-Met	40	0.429	0.126^{cd}	0.257^{a}	0.164^{abc}
SEM		0.031	0.005	0.011	0.010
P value					
Source		> 0.05	0.01	0.0003	0.010
Dose		0.001	> 0.05	0.0001	> 0.05
Source*Dose		> 0.05	> 0.05	0.0477	0.0177
Regression Analysis					
Comparison of Slope		0.630	0.333	0.030	0.630

a-d : $P < 0.05$.

Table 6.6A. Effect of dose on tissue Se concentration of broiler chicks at 7 days of age injected with Se at 10 days of incubation.

Dose, μg Se/egg	Liver Se μg/g	Lung Se μg/g	Heart Se μg/g	Breast Se μg/g
0	0.210[c]	0.126	0.139[c]	0.137
2.5	0.284[b]	0.135	0.161b[c]	0.143
5	0.308[b]	0.131	0.166[b]	0.143
10	0.292[b]	0.128	0.167[b]	0.162
20	0.326a[b]	0.132	0.200[a]	0.157
40	0.400[a]	0.121	0.212[a]	0.143
SEM	0.022	0.003	0.011	0.004
P value	0.001	> 0.05	0.000	> 0.05

a-c : $P < 0.05$.

Table 6.6B. Effect of Se source on tissue Se concentration of broilers at 7 days of age injected with Se at 10 days of incubation.

Se Source	Liver Se μg/g	Lung Se μg/g	Heart Se μg/g	Breast Se μg/g
Na_2SeO_3	0.294	0.125[b]	0.158[b]	0.139[b]
Se-Met	0.313	0.133[a]	0.190[a]	0.156[a]
SEM	0.126	0.002	0.0	0.007
P value	0.321	0.009	0.000	0.010

a : $P < 0.05$.

Table 6.7. Tissue Se concentration (wet basis, μg/g) of broiler chicks 14 at days of age injected with Se at 10 days of incubation.

Se Source	Dose μg Se/egg	Liver Se μg/g	Lung Se μg/g	Heart Se μg/g	Breast Se μg/g
Na_2SeO_3	0	0.229	0.162^{de}	0.120	0.108
Na_2SeO_3	2.5	0.228	0.168^{cde}	0.125	0.106
Na_2SeO_3	5	0.240	0.162^{de}	0.130	0.105
Na_2SeO_3	10	0.221	0.158^{e}	0.130	0.105
Na_2SeO_3	20	0.245	0.169^{bcde}	0.134	0.121
Na_2SeO_3	40	0.251	0.162^{de}	0.145	0.124
Se-Met	0	0.229	0.162^{de}	0.120	0.108
Se-Met	2.5	0.284	0.181^{ab}	0.112	0.121
Se-Met	5	0.256	0.169^{bcde}	0.143	0.108
Se-Met	10	0.233	0.178^{abc}	0.135	0.119
Se-Met	20	0.233	0.182^{a}	0.154	0.125
Se-Met	40	0.225	0.174^{abcd}	0.139	0.118
SEM		0.021	0.003	0.011	0.008
P value					
Source		> 0.05	0.0001	> 0.05	> 0.05
Dose		> 0.05	0.0234	> 0.05	> 0.05
Source*Dose		> 0.05	> 0.05	> 0.05	> 0.05
Regression Analysis					
Comparison of Slope		0.119	0.119	0.964	0.246

a-e : $P < 0.05$.

Table 6.8. Tissue Se concentration (wet basis, μg/g) of broiler chicks at 21 days of age injected with Se at 10 days of incubation.

Se Source	Dose	Liver Se μg/g	Lung Se μg/g	Heart Se μg/g	Breast Se μg/g
Na_2SeO_3	0	0.250	0.135^{de}	0.135	0.100
Na_2SeO_3	2.5	0.266	0.140^{cde}	0.142	0.102
Na_2SeO_3	5	0.242	0.135^{de}	0.142	0.105
Na_2SeO_3	10	0.263	0.131^{e}	0.141	0.115
Na_2SeO_3	20	0.272	0.142^{bcde}	0.138	0.105
Na_2SeO_3	40	0.240	0.135^{de}	0.146	0.111
Se-Met	0	0.250	0.135^{de}	0.135	0.106
Se-Met	2.5	0.274	0.153^{ab}	0.140	0.110
Se-Met	5	0.264	0.141^{bcde}	0.151	0.114
Se-Met	10	0.265	0.150^{abc}	0.140	0.103
Se-Met	20	0.289	0.155^{a}	0.154	0.103
Se-Met	40	0.298	0.147^{abcd}	0.157	0.164
SEM		0.016	0.004	0.006	0.005
		P value			
Source		> 0.05	0.0002	> 0.05	> 0.05
Dose		> 0.05	0.0342	> 0.05	> 0.05
Source*Dose		> 0.05	> 0.05	> 0.05	> 0.05
Regression Analysis					
Comparison of Slope		0.100	0.459	0.331	0.720

a-e : $P < 0.05$.

Table 6.9A. Effect of Se source on lung Se concentration on broiler chicks at 14 and 21 days of age, injected with Se at 10 days of incubation.

Se Source	Lung Se, µg/g	
	Day 14	Day 21
Na_2SeO_3	0.164^{b}	0.136^{b}
Se-Met	0.174^{a}	0.147^{a}
SEM	0.003	0.002
P value	0.0001	0.034

a-b : $P < 0.05$.

Table 6.9B. Effect of Se dose administered on broiler chicks at 14 and 21 days of age after injecting Se at 10 days of incubation.

Dose, µg Se/egg	D14 Lung Se µg/g	D21 Lung Se µg/g
0	0.162^{b}	0.135^{c}
2.5	0.174^{a}	0.147^{ab}
5	0.165^{b}	0.138^{bc}
10	0.168^{ab}	0.141^{abc}
20	0.176^{a}	0.148^{a}
40	0.168^{ab}	0.141^{abc}
SEM	0.003	0.003
P value	0.023	0.034

a-c : $P < 0.05$.

Table 6.10. Effect of Se injection at on tissue lipid peroxidation at Day 20 of incubation.

Se Source	Dose μg Se/egg	Thiobarbituric acid reactive substances, AU[1]			
		Liver	Lung	Heart	Breast
Na_2SeO_3	0	0.004	0.0120^{b}	0.0385^{b}	0.0200^{bc}
Na_2SeO_3	2.5	0.003	0.0130^{a}	0.0375^{b}	0.0165^{cd}
Na_2SeO_3	5	0.001	0.0088^{f}	0.0400^{b}	0.0225^{b}
Na_2SeO_3	10	0.001	0.0105^{e}	0.0265^{c}	0.0065^{f}
Na_2SeO_3	20	0.001	0.0105^{e}	0.0355^{b}	0.0120^{de}
Na_2SeO_3	40	0.001	0.0120^{b}	0.0540^{a}	0.0155^{cd}
Se-Met	0	0.004	0.0125^{c}	0.0385^{b}	0.0200^{bc}
Se-Met	2.5	0.003	0.0020^{j}	0.0175^{de}	0.0200^{bc}
Se-Met	5	0.001	0.0115^{d}	0.0245^{cd}	0.0100^{ef}
Se-Met	10	0.001	0.0075^{i}	0.0135^{c}	0.0330^{a}
Se-Met	20	0.001	0.0080^{h}	0.0270^{c}	0.0120^{de}
Se-Met	40	0.0001	0.0085^{g}	0.0210^{cde}	0.0140^{de}
SEM		0.0007	0.0001	0.0025	0.0017
		P value	*P* value	*P* value	*P* value
Source		> 0.05	<0.0001	<0.0001	0.0178
Dose		0.0053	<0.0001	0.0001	0.0031
Source*Dose		> 0.05	<0.0001	0.0007	<0.0001

[1] Absorption Units at 530 nm
a-j : $P < 0.05$.

Table 6.11A. Effect of Se source on tissue lipid peroxidation at Day 20 of incubation.

Se Source	Thiobarbituric acid reactive substances, AU[1]			
	Liver	Lung	Heart	Breast
Na_2SeO_3	0.0022	0.011[a]	0.039[a]	0.015[b]
Se-Met	0.0019	0.008[b]	0.024[b]	0.018[a]
SEM	0.6213	0.000	0.001	0.007
P value	> 0.05	<0.001	<0.001	0.018

[1]Absorption Units at 530 nm.
a-b : $P < 0.05$.

Table 6.11B. Effect of Se dose on tissue lipid peroxidation at Day 20 of incubation.

Dose, µg Se/egg	Thiobarbituric acid reactive substances, AU[1]			
	Liver	Lung	Heart	Breast
0	0.0045[a]	0.0123[a]	0.0385[a]	0.0200[a]
2.5	0.0032[a]	0.0075[e]	0.0275[c]	0.0183[abc]
5	0.0010[b]	0.0101[b]	0.0323[b]	0.0163[bc]
10	0.0012[b]	0.0090[d]	0.0200[d]	0.0198[ab]
20	0.0012[b]	0.0093[c]	0.0313[bc]	0.0120[cd]
40	0.0010[b]	0.0103[b]	0.0375[ab]	0.0148[d]
SEM	0.0060	0.0001	0.0020	0.0020
P value	0.005	< 0.001	< 0.001	0.003

[1]Absorption Units at 530 nm.
a-e : $P < 0.05$

Table 6.12. Effect of *in ovo* Se injection on the antibody response to SRBC in 3-d old broiler chicks. The values are reported in log_2 (2 log reduction in titers).

		Days post primary injection			
		0	7	13	18[1]
	Days in age	3	10	16	22
Source	Dose µg Se/egg	Total Anti-SRBC Antibody Response			
Na_2SeO_3	0	0	1	1	5.5
Na_2SeO_3	2.5	0	2	0.5	7.5
Na_2SeO_3	5	0	4	2.5	7
Na_2SeO_3	10	0	3	0.5	6.5
Na_2SeO_3	20	0	3.5	1	7
Na_2SeO_3	40	0	2	1	7.5
Se-Met	0	0	1	1	5.5
Se-Met	2.5	0	2.25	1	5
Se-Met	5	0	3.5	0	6.5
Se-Met	10	0	2.5	1	8
Se-Met	20	0	2	1	7
Se-Met	40	0	3	0.5	5.5
		IgG Anti-SRBC Antibody Response			
Na_2SeO_3	0	0	0	1	2
Na_2SeO_3	2.5	0	0	0	1.5
Na_2SeO_3	5	0	0	1	6
Na_2SeO_3	10	0	0	0	1.5
Na_2SeO_3	20	0	0	0	2
Na_2SeO_3	40	0	0	0.5	1
Se-Met	0	0	0	1	2
Se-Met	2.5	0	0	0.5	0
Se-Met	5	0	0	0	1.5
Se-Met	10	0	0	0	1.5
Se-Met	20	0	0	0	2
Se-Met	40	0	0	0	2
		IgM Anti-SRBC Antibody Response			
Na_2SeO_3	0	0	1	0	3.5
Na_2SeO_3	2.5	0	2	0.5	6
Na_2SeO_3	5	0	4	1.5	1
Na_2SeO_3	10	0	3	0.5	5
Na_2SeO_3	20	0	3.5	1	5
Na_2SeO_3	40	0	2	0.5	6.5
Se-Met	0	0	1	0	3.5
Se-Met	2.5	0	2.25	0.5	5
Se-Met	5	0	3.5	0	5
Se-Met	10	0	2.5	1	6.5
Se-Met	20	0	2	1	5
Se-Met	40	0	3	0.5	3.5

[1]18 days post primary SRBC injection or 4 days post- secondary SRBC injection.

Table 6.13A. Effect of *in ovo* selenium injection at 7 dpi[1] SRBC immune challenge.

	----------*P* values----------		
	Total	IgG[2]	IgM
Source	0.940	-	0.940
Dose	0.235	-	0.235
Source*Dose	0.601	-	0.601

[1]dpi = days post primary injection.
[2] IgG = Not detected.

Table 6.13B. Effect of *in ovo* selenium injection at 13 dpi[1] SRBC immune challenge.

	----------*P* values----------		
	Total	IgG	IgM
Source	0.172	0.071	0.522
Dose	0.598	0.0003	0.555
Source*Dose	0.001	0.0002	0.138

[1]dpi = days post primary injection.

Table 6.13C. Effect of *in ovo* selenium injection at 4 dp2[1] SRBC immune challenge.

	----------*P* values----------		
	Total	IgG	IgM
Source	0.161	0.780	0.332
Dose	0.492	0.174	0.121
Source*Dose	0.199	0.068	0.026

[1]dp2 = days post- secondary injection.

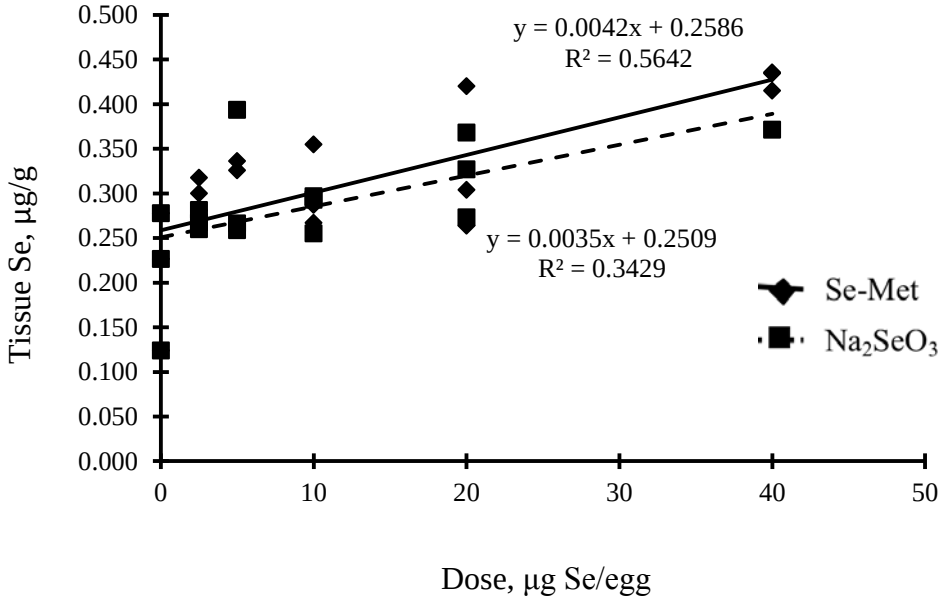


Figure 6.1A. Liver Se concentration (wet basis, µg/g) of broiler chicks at 7 days of age.

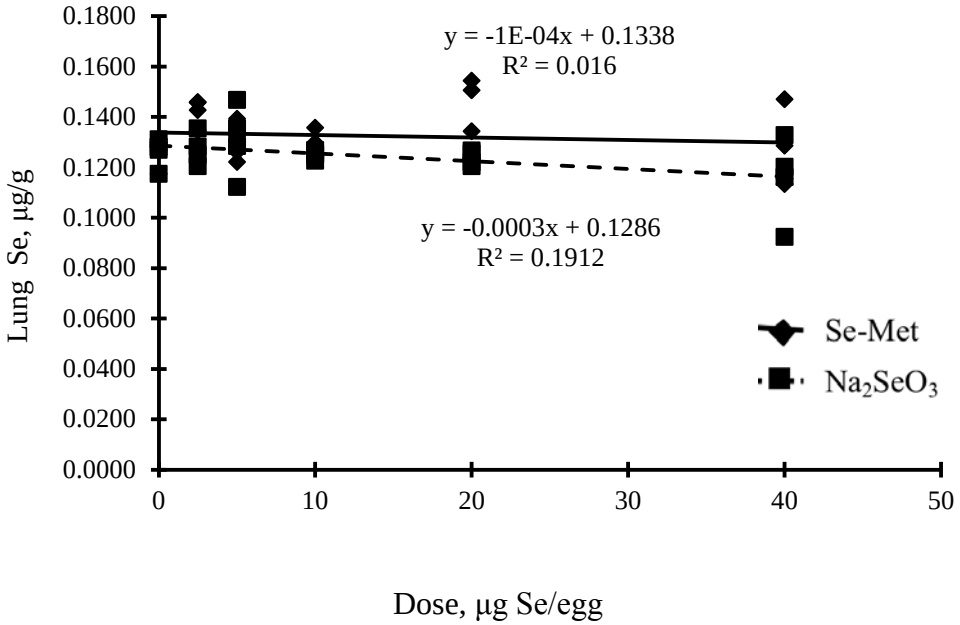


Figure 6.1B. Lung Se concentration (wet basis, µg/g) of broiler chicks at 7 days of age.

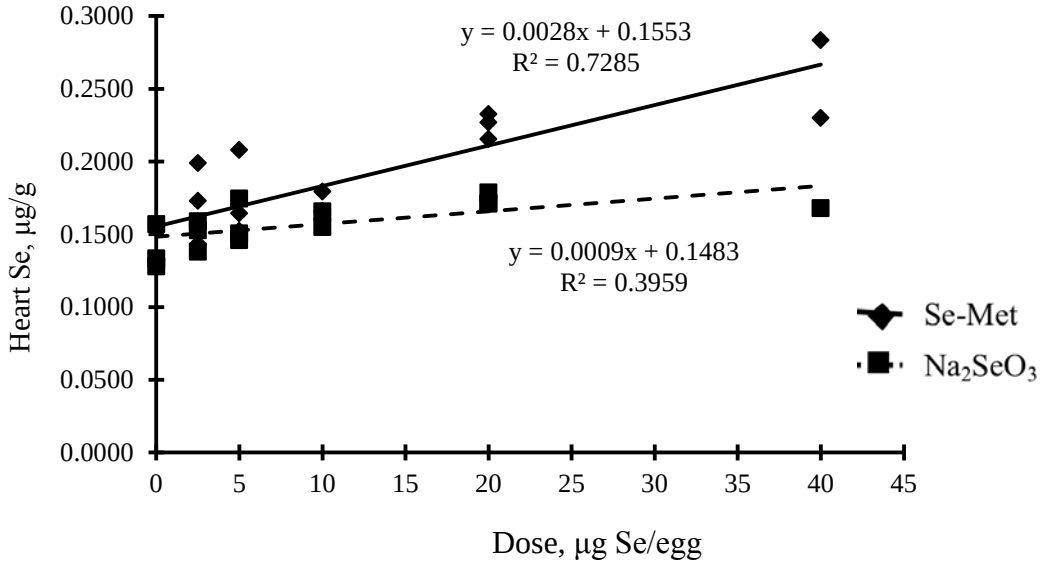


Figure 6.1C. Heart Se concentration (wet basis, µg/g) of broiler chicks at 7 days of age.

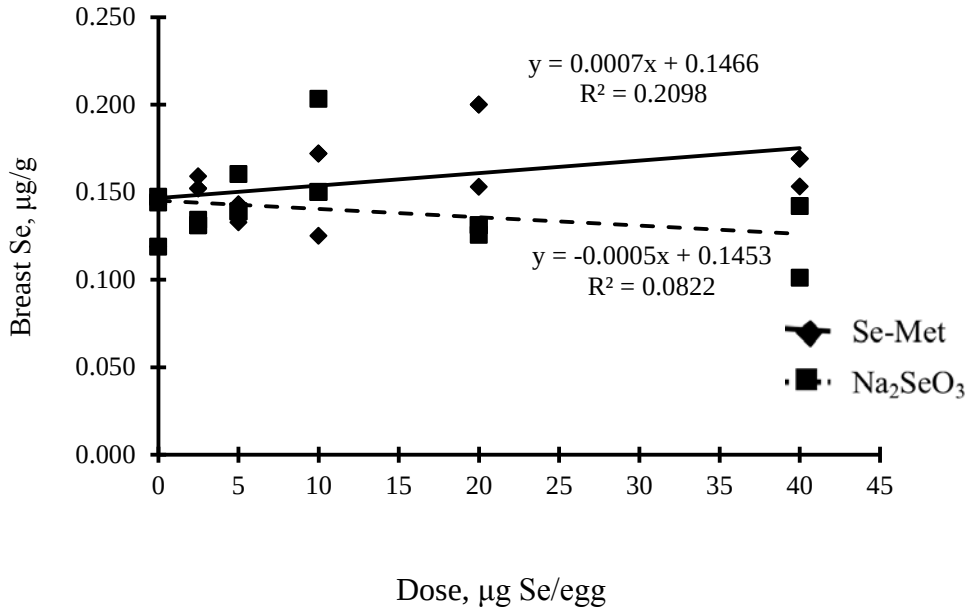


Figure 6.1D. Breast Muscle Se concentration (wet basis, µg/g) of broiler chicks at 7 days age

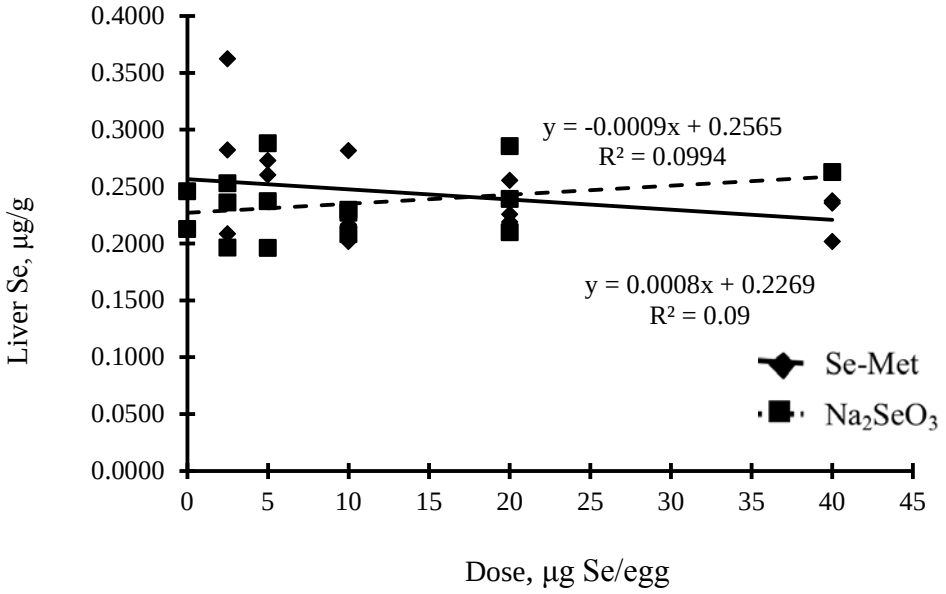


Figure 6.2A. Liver Se concentration (wet basis, µg/g) of broiler chicks at 14 days of age.

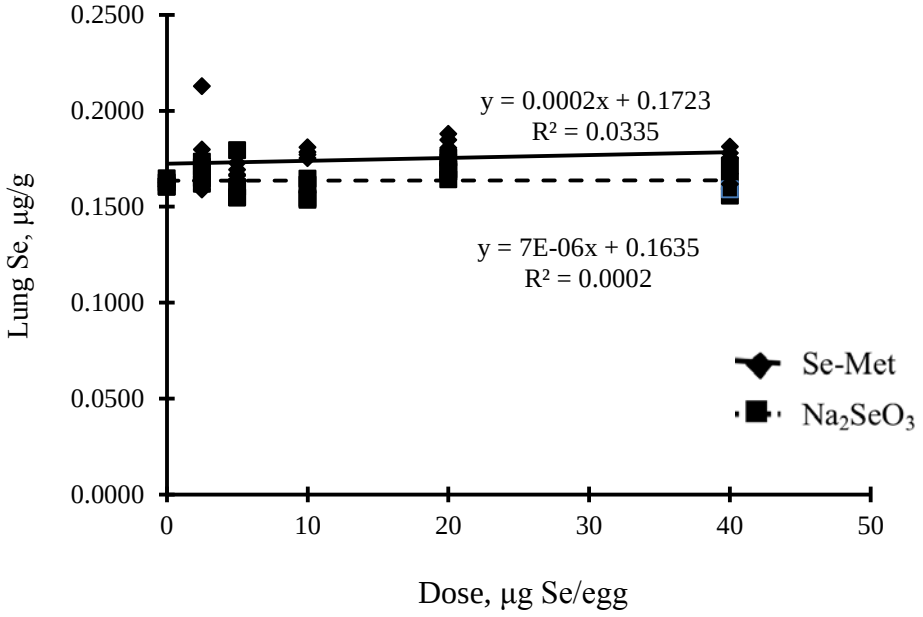


Figure 6.2B**.** Lung Se concentration (wet basis, µg/g) of broiler chicks at 14 days of age.

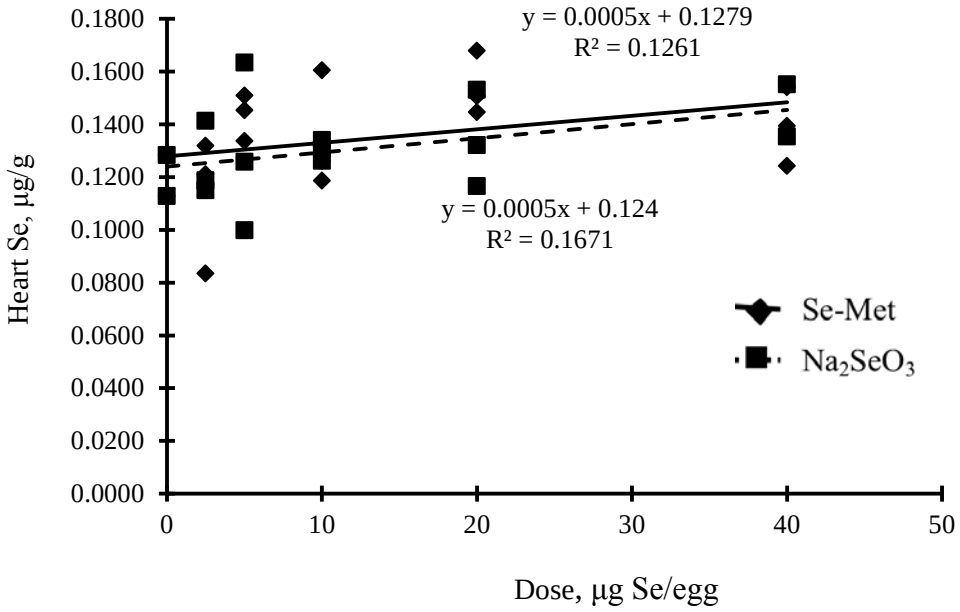


Figure 6.2C. Heart Se concentration (wet basis, µg/g) of broiler chicks at 14 days of age.

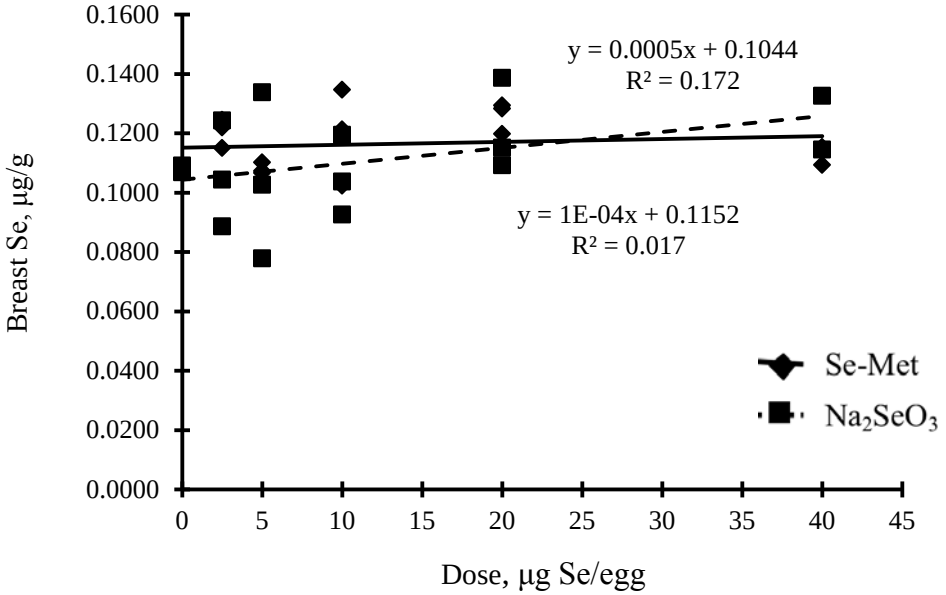


Figure 6.2D. Breast Muscle Se concentration (wet basis, µg/g) of broiler chicks at 14 days of age.

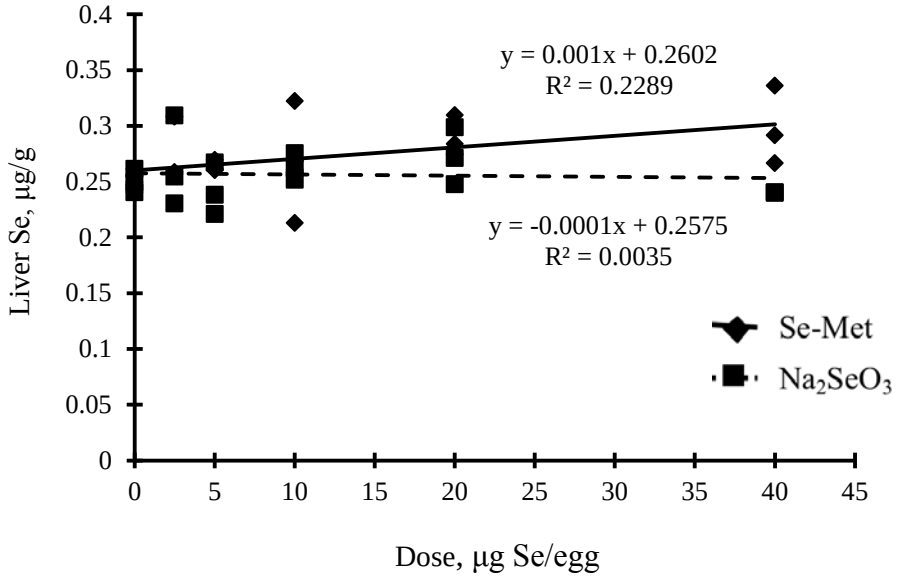


Figure 6.3A. Liver Se concentration (wet basis, µg/g) of broiler chicks at 21 days of age.

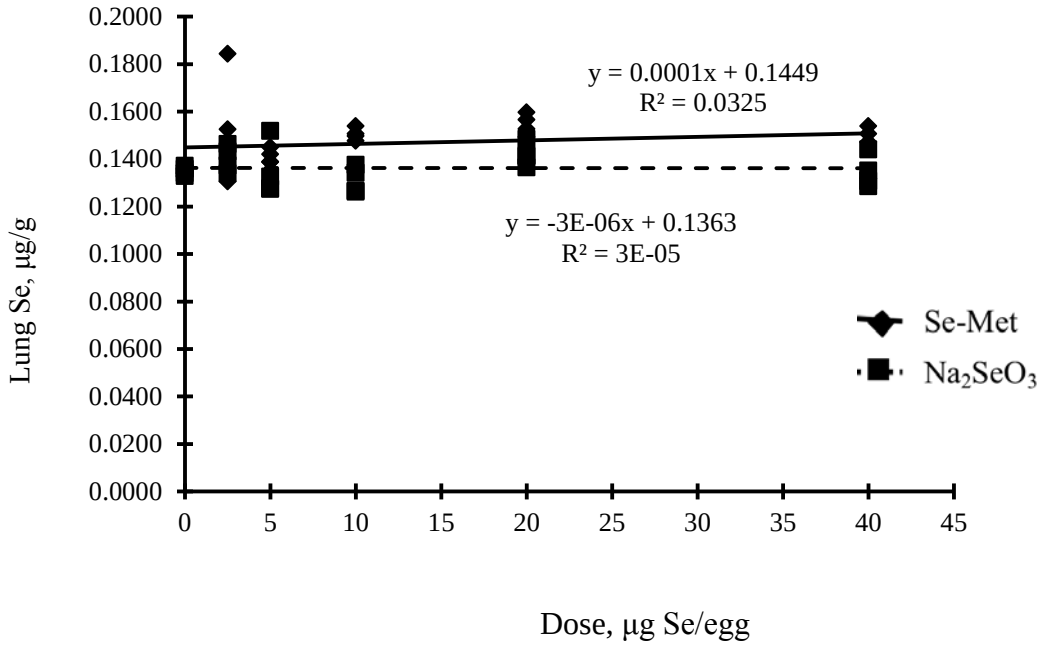


Figure 6.3B. Lung Se concentration (wet basis, µg/g) of broiler chicks at 21 days of age.

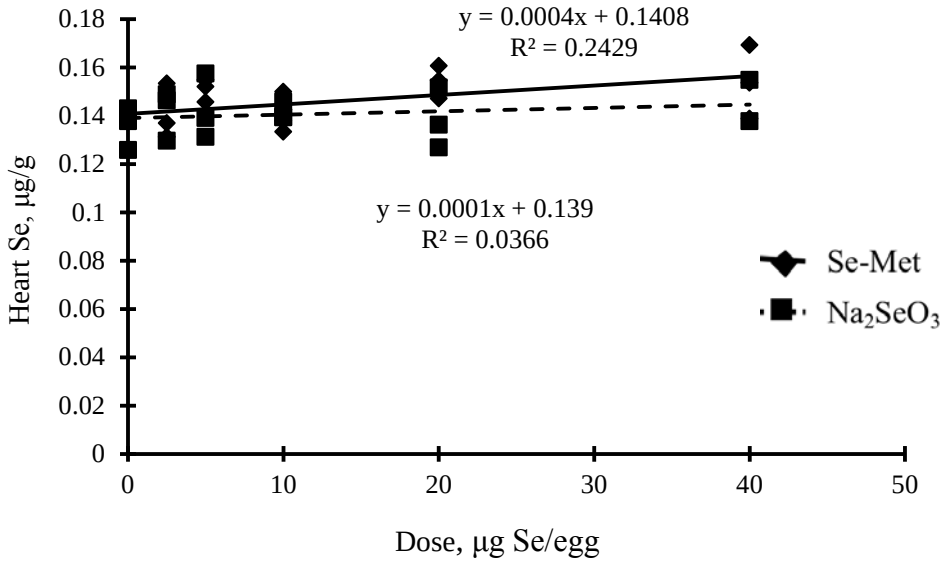


Figure 6.3C. Heart Se concentration (wet basis, µg/g) of broiler chicks at 21 days of age.

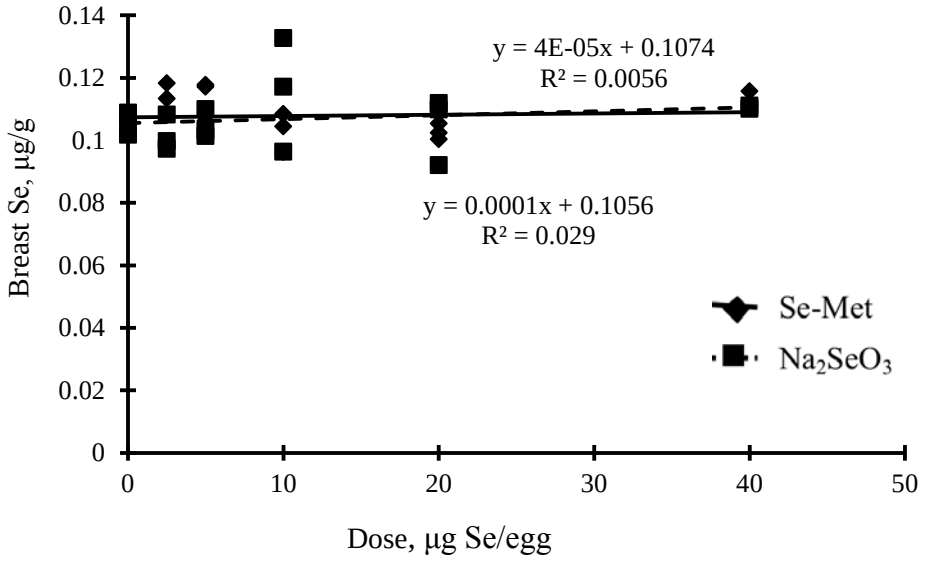


Figure 6.3D. Breast Muscle Se concentration (wet basis, µg/g) of broiler chicks at 21 days of age.

CHAPTER 7: EFFECT OF SELENIUM SUPPLEMENTATION OF BREEDER HEN DIETS ON TISSUE SE CONCENTRATIONS IN PROGENY

INTRODUCTION

Selenium is an essential trace mineral that is required by animals including poultry (NRC, 1994) for overall health and growth performance. The essentiality of Se was established when Schwarz and Foltz (1957) reported that Se is required nutrient that can prevent liver necrosis in rats. Then in 1973, (Rotruck *et al.*) defined the specific role of Se as being part of the structural component of glutathione peroxidase (GSH-Px). It has been shown that GSH-Px prevent lipid peroxidation by converting lipid hydroperoxides to non-toxic alcohol and water. As free radicals are normally produced by the biological system, it is imperative that these radicals be quenched to prevent potential cellular damage. Cellular damages if left unchecked can often lead to pathological conditions. In poultry diets, Se is reportedly added to prevent exudative diathesis, pancreatic fibrosis, gizzard and heart myopathies, immune deficiency, reduced hatchability, reduced fertility and poor feathering among others (Cantor *et al.*, 1975a, Scott *et al.*, 1957; Schwarz *et al.*, 1957; Patterson *et al.*, 1957; Noguchi *et al.*, 1973; Cantor *et al.*, 1975b; Thompson and Scott, 1969; Gries and Scott 1972; Cantor *et al.*, 1982b, Scott *et al.*, 1967; Arthur *et al.*, 2003; Combs, 1994; Latshaw and Osman, 1974; Cantor and Scott, 1974; Edens, 1996).

The use of Se supplements for poultry diets was approved by the US FDA in 1974. The only two compounds of Se that were permitted were sodium selenite and sodium selenate. Then, in 2000, the FDA approved the use of an organic form of Se in chicken diets, namely selenium yeast which was described by Kelly and Power (1995). The major Se component of Se yeast is in the form of seleno-methionine, which is also chiefly found in cereals and grain used as feed ingredients. With the approval of the use of organic Se in poultry as well as other livestock, research studies have shown the effect of feeding organic Se as being superior in tissue Se accumulation than when $NaSe_2O_3$ is added to the diet. Although, the concentration of Se found in cereals and grain varies according to geographical location in the US (Kubota *et al.*, 1967). Similarly, Cantor (1997) conducted a survey on Se levels of corn and soybean meal used as feed ingredient from

several US states. He reported that the average Se levels of corn ranged from 0.024 to 0.42 ppm while the average Se levels for soybean meal was 0.06 to 0.08 ppm. Thus, the concentration of Se from corn and soybean meal varies from state to state.

The essentiality of Se in the hen's diet is important for egg production and hatchability as well as for the overall performance of progeny chicks as shown by Cantor and Scott (1974). Thus, maternal nutrition is critical to the growth and development of the incubating embryo. Chickens are considered as precocial species; such that all the required nutrients must be supplied to the egg prior to lay. Since embryos develop outside of the hen after the egg has been laid, it no longer has access to maternal nutrition except to what has been transferred to the yolk prior to lay.

In a study conducted by Paton *et al.* (2002), it was shown that organic Se (Se yeast) as well as inorganic Se when added to the maternal diet could be transferred to the egg and subsequently to the embryo. This study showed that 0.3 mg/kg Se as Se yeast was transferred to the egg and the embryo significantly higher than $NaSe_2O_3$ on equal amount. These authors showed that deposition of selenium linearly increases with increasing Se concentration particularly with organic selenium. Furthermore, the greatest increase in the Se embryo concentration was between days 10-15 of incubation. Surai (2000) conducted a similar study showing that addition of Se yeast in the broiler breeder diet increased the Se status of progeny tissues. These results lead us to the question on what happens to the tissue Se levels of embryo when they are hatched and grown for at least three weeks and fed a low Se diet.

The objective of this experiment was to evaluate the effect of dietary Se supplementation of broiler breeder hens on the tissue concentration of progeny chicks fed a low Se diet. In addition, it was determined how long will enhanced tissue Se levels be observed in chicks-fed low Se levels when their dams are fed Se yeast.

MATERIALS AND METHODS

Chicks and Diet

Chick Study

Chicks (straight run) used for this experiment were obtained from broiler breeders that were fed the following experimental diets; basal diet with no Se added and basal diet with 0.3 ppm Se either as $NaSe_2O_3$ or Se yeast. Chicks were housed in mesh wire-floored pullet starter cages (61 cm x 51 cm x 36 cm) with temperature of 31^{o}C for the first week and 27^{o}C for the remainder of the study. Each cage was provided with one feeder and two adjustable nipple drinkers. For the duration of the study, all chicks were fed a low Se broiler starter diets shown in Table 6.1. Water and feed were provided *ad libitum.*

A completely randomized treatment arrangement was used for this experiment For Trial 1, there were 16 replicates of five chicks/cage based on maternal dietary treatment and for Trial 2, four replicates cages of five chicks per maternal dietary treatment were used.

Animal Welfare

This experiment was approved by the University of Kentucky Institutional Animal Care and Use Committee.

Tissue Sampling

Blood was collected via wing vein puncture and the collected blood was transferred to a vaccutainer containing heparin to prevent coagulation. For plasma extraction, blood samples were centrifuged for 15 mins at 2500 x g. Tissues samples such as liver and breast muscle as well as the blood were collected at days 7,14 and 21 or 22. Tissue samples were collected from two birds (pooled) from each of the four replicates per treatment whereas blood samples were collected intravenously from three birds from each of the four replicates/treatment. Samples were stored at -20C until analysis.

Table 7.1. Composition of broiler starter diet fed to all chicks.

Feed Ingredient		Calculated Analysis	
Corn	56.25%	Metabolizale Energy	1.39 Mcal/kg
Soybean Meal	36.00%	Crude Protein	22.4%
Corn oil	3.50%	Calcium	1.01%
Salt	0.45%	Available Phosphorus	0.45%
Methionine DL 99%	0.19%	Total Sulfur Amino Acid	0.90%
Vitamin mix*	0.25%		
Limestone	1.35%		
Dicalcium Phosphate	1.76%		
Mineral mix (no Se)	0.25%		

* Provided 33 IU Vit. E/kg

Laboratory Analysis

Plasma, blood, liver and breast muscle Se were analyzed using the fluorometric assay following nitric/perchloric acid digestion according to the procedures of Olson *et al.*, (1975) with modifications by Cantor and Tarino (1982).

Statistical Analyses

All the data gathered from the experiments were subjected to ANOVA using the General Linear Model procedures for a completely randomized design experiment using Statistical Analysis System (SAS) software. Differences among means were separated by the test of least significant difference. A probability of $P < 0.05$ was required for significance.

RESULTS AND DISCUSSION

The analyses for diets used for the current study showed that the semi-purified breeder diet with no Se added contained μg/g Se while the chick starter diet, indicated that it contained 0.035 μg/g Se.

The effect of dietary addition of Se to maternal diet on the egg Se concentration is shown on Table 7.2. Dietary supplementation of Se significantly increased the egg Se concentration of eggs from Se compared to basal (no Se is added) treatment. While both

Se sources increased the egg Se, the addition of resulting egg Se level from Se yeast was 37% greater than from $NaSe_2O_3$ ($P < 0.001$). This result is in agreement with earlier studies on Se transfer to eggs (Paton *et al.*, 2002), in which graded additions of Se as Se yeast or Na_2SeO_3 linearly increased egg Se concentrations, Surai (2000) also reported similar findings, wherein the addition of 0.02 or 0.04 mg/kg Se as Se yeast to a breeder diet led to increase Se transfer to the yolk and albumen. This is important because, more Se in the yolk may be available for transport to embryonic tissues, hence, possibly improving antioxidant capacity.

Results for Trial 1 are shown on Table 7.3 Adding Se as $NaSe_2O_3$ or Se yeast to the breeder diet significantly increased plasma Se and liver Se at Day 1, blood Se at Day 13 and liver Se at Day 22 in chicks, compared to the control treatment. Compared with $NaSe_2O_3$, supplementing Se yeast resulted in significantly higher liver Se at Day1 (0.52 *vs* 0.32 μg/g).

In Trial 2, Se supplementation of the breeder diet with either $NaSe_2O_3$ or Se yeast significantly elevated blood, liver and breast muscle Se of chicks at Day 1. Se yeast resulted in significantly greater Se levels compared with $NaSe_2O_3$ (Figures 7.1, 7.2 and 7.3). Se yeast but not $NaSe_2O_3$ also elevated blood and liver Se in chicks at Day 7. By Day 14, Se levels in blood, liver and breast muscle of chicks were similar for all treatments. Seleno-methionine is major component of Se yeast (Kelly and Power, 1995), Cantor and Tarino (1982) compared the effects of supplementing turkey diets with Se-Met and $NaSe_2O_3$ on tissue Se. Seleno-methionine resulted in higher blood and erythrocyte Se concentrations. Payne and Southern (2005b) reported that feeding broiler chicks with organic Se and Na_2SeO_3 resulted plasma Se levels of 0.160 and 0.137 μg/g respectively. Our results for breast muscle Se concentration (Figure 7.3) are comparable with several earlier studies showing a significant increase in tissue Se accumulation due to supplements of Se yeast (Payne and Southern 2005b) or Se-Met (Cantor *et al.*, 1982), compared with Na_2SeO_3.

The highest increase in Se concentration was observed on d 1 when breast muscle Se for Se yeast treatment was 2x higher than that for $NaSe_2O_3$ (0.18 vs 0.09 μg/g). This is comparable to the results obtained by Pappas *et al.* (2006), who concluded that broilers breeders fed a high Se diet had progeny with higher liver and brain Se. Increased Se

status of hatched chicks persisted up to 14 days. Earlier on, Pappas *et al.*, (2005) conducted a study in which progeny chicks hatched from breeders whose diets were supplemented with Se yeast (0.419 μg Se/g feed) were fed a low Se diet for 4 weeks. They observed that at day of hatch, breast Se was significantly higher in offspring (4.3 times) from Se yeast supplemented hens than from control hens. The breast Se level dropped by d 7 but was still significantly higher than control birds up to 28 days. This trend was also seen in whole blood samples. Higher liver Se persisted up to 21 days in comparison with the other tissues.

In the current study, Se supplementation of breeder hen diets resulted in a significantly greater maternal transfer of Se to progeny chicks. The results of this study showed that maternal supplementation of Se not only influences Se status of chicks at hatch but also during growth. This was evident by the differences in the tissue concentration of Se, while the chicks were fed low Se diets.

Taken together, the results of this experiment showed that the progeny tissue Se levels in blood or plasma and tissues were all dependent on the amount and source of Se added to the maternal diet. The addition of adequate Se in the hen's diet is prerequisite to elevating the Se status of embryo, and, thus, the chicks. Moreover, the addition of Se yeast further elevates tissue Se concentration compared with $NaSe_2O_3$. Finally dietary supplementation of Se is essential to prevent the possible Se deficiency that may occur in regions where Se levels in the soil and feed ingredient is low.

Table 7.2. Effect of Se supplementation on broiler breeder hen diets on egg Se levels.

Breeder Diet	Egg Se, μg/g, wet basis	% increase (*vs* Na_2SeO_3)
Basal, no added Se	0.041[c]	
Basal + 0.3 ppm Se as $NaSe_2O_3$	0.185[b]	
Basal + 0.3 ppm Se as Se Yeast	0.253[a]	37
SEM	0.017	
P value	0.000	

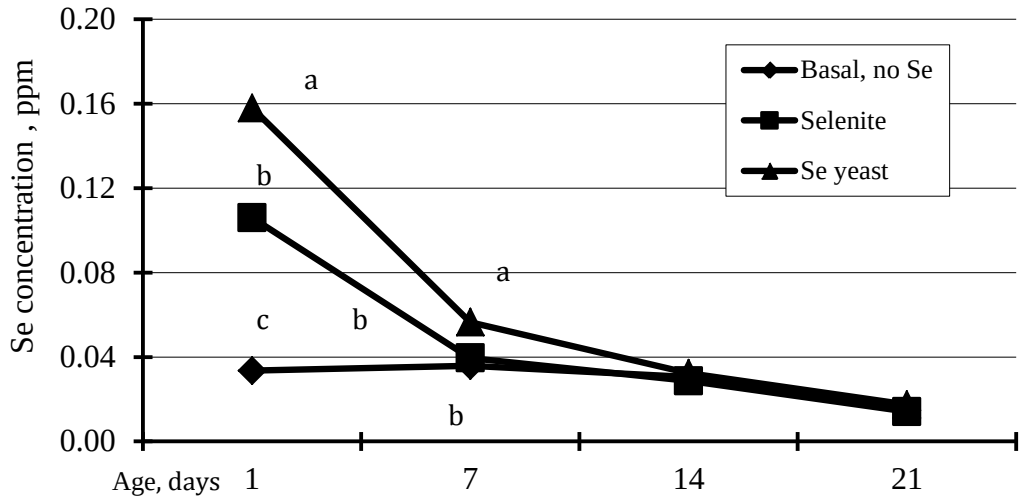
a-c : $P < 0.05$

Table 7.3. Effect of Se supplementation of breeder hen diets on tissue Se concentration (wet basis, μg/g) of progeny chicks. (Trial 1)

Breeder Diet Se Source and Level	Plasma Day 1	Liver Day 1	Blood Day 13	Liver Day 22
Basal , no Se added	0.018[b]	0.12[c]	0.04[b]	0.08[b]
Basal + 0.3 ppm Se as $NaSe_2O_3$	0.100[a]	0.32[b]	0.05[a]	0.11[a]
Basal + 0.3 ppm Se as Se Yeast	0.100[a]	0.52[a]	0.06[a]	0.10[a]
SEM	0.009	0.038	0.003	0.006
P value	0.006	0.001	0.029	0.012

a-c : $P < 0.05$

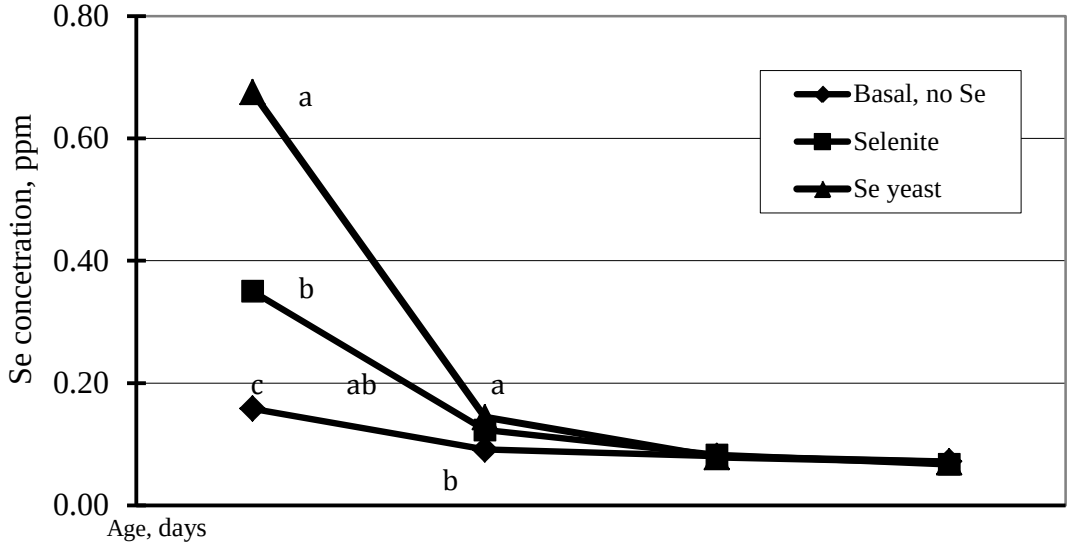
Figure 7.1. Effect of Se supplementation on of breeder hen diets on blood Se concentration (wet basis, μg/g) of progeny chicks fed a low Se diet. (Trial 2)



Dietary Treatment	Day 1	Day 7	Day 14	Day 21
Basal, no Se added	0.03[c]	0.04 [b]	0.03	0.01
Basal + 0.3 ppm Se as $NaSe_2O_3$	0.11 [b]	0.04 [b]	0.03	0.01
Basal + 0.3 ppm Se as Se yeast	0.16 [a]	0.06 [a]	0.03	0.02
SEM *P* value	0.004 <0.0001	0.004 0.012	0.002 0.140	0.001 0.079

a-c: $P < 0.05$

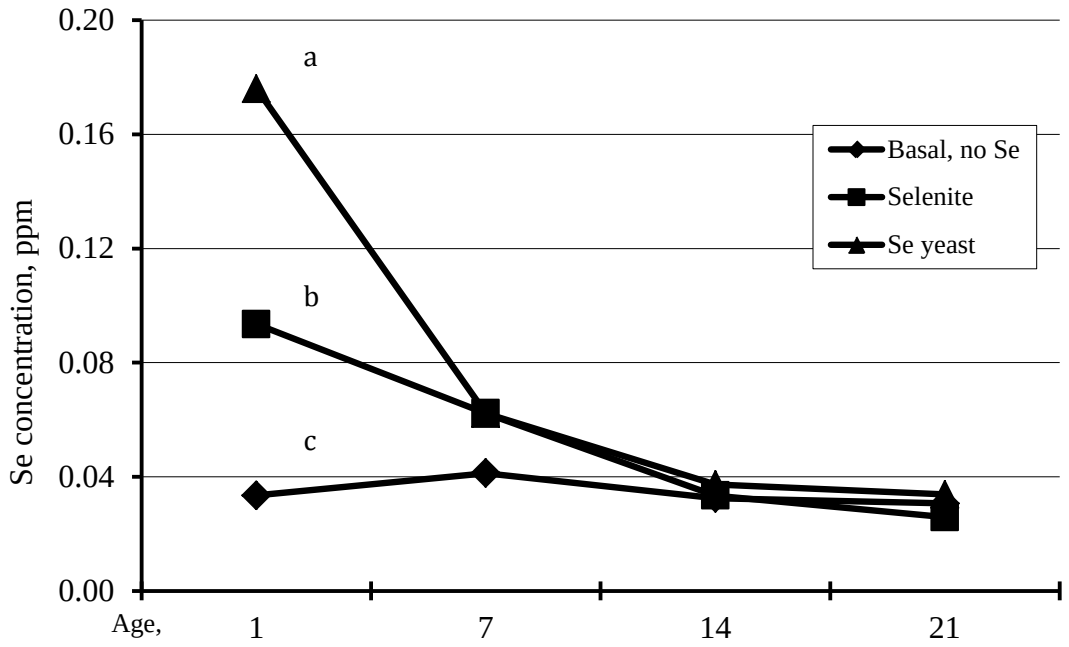
Figure 7.2. Effect of Se supplementation of breeder hen diets on liver Se concentration (wet basis, μg/g) of progeny chicks fed a low Se diet. (Trial 2)



Dietary Treatment	Day 1	Day 7	Day 14	Day 21
Basal, no Se added	0.16^{c}	0.09 ^{b}	0.08	0.07
Basal + 0.3 ppm Se as $NaSe_2O_3$	0.35 ^{b}	0.12 ^{ab}	0.08	0.07
Basal + 0.3 ppm Se as Se yeast	0.68 ^{a}	0.24 ^{a}	0.08	0.07
SEM	0.036	0.011	0.006	0.005
P value	<0.0001	0.021	0.905	0.783

a-c : $P < 0.05$

Figure 7.3. Effect of Se supplementation of breeder hen diets on breast muscle Se (wet basis, μg/g) of progeny chicks fed a low Se diet (Trial 2).



Dietary Treatment	Day 1	Day 7	Day 14	Day 21
Basal, no Se added	0.03[c]	0.04	0.03	0.03
Basal + 0.3 ppm Se as $NaSe_2O_3$	0.09 [b]	0.06	0.03	0.03
Basal + 0.3 ppm Se as Se yeast	0.18 [a]	0.06	0.04	0.03
SEM *P* value	0.033 <0.0001	0.006 0.075	0.002 0.242	0.003 0.181

a-c : $P < 0.05$

CHAPTER 8: CONCLUSIONS

The purpose of this research was to investigate the effects of in ovo Se injection of incubating White Leghorn and broiler eggs as a possible means of enhancing Se status of chicks during embryonic and post-hatch development. In the experiments, graded doses of Se-Met and Na_2SeO_3 were used to inject the yolk of fertile eggs after 10 d of incubation and determine the fate of Se once it has been absorbed and deposited into different embryonic tissues; its effects on embryo viability, lipid peroxidation, immune response and post hatch development.

The first study (Chapter 3), using a solution containing 2.5, 5, 10 or 20 µg Se/g Se as Se-Met or Na_2SeO_3 dissolved in PBS were injected into the yolk of White Leghorn fertile eggs. Embryo viability values for Se-Met were 97, 94, 90 and 83%, respectively, whereas the respective values for eggs treated with Na_2SeO_3 were 87, 94, 74 and 87%. Sham-control eggs injected with PBS had 94% embryo viability. Tissue Se levels increased as Se dose injected increased. Embryonic liver and lung from eggs injected with Na_2SeO_3 had higher Se concentration than from eggs injected with Se-Met. There was no difference observed in the heart Se levels from eggs injected with either Se.

In the second study (Chapter 4), White Leghorn fertile eggs were injected with 0, 20, 40 or 60 µg Se/egg Se-Met or Na_2SeO_3 dissolved in PBS. Injecting fertile eggs up to 60 µg Se did not negatively affect embryo livability. Embryos from eggs injected with Se-Met had higher viability than from eggs injected with Na_2SeO_3. Linear increases in all tissues were obtained with increasing doses of Se. However, the change in heart and breast muscle Se concentrations was minimal above the 40 µg dose when Na_2SeO_3 was used. Linear regression coefficients for tissue Se vs. dose for all tissues were significantly greater for Se-Met than Na_2SeO_3.

In the third study (Chapter 5), 2.5, 5, 10, 20 or 40 µg Se/g Se as Se-Met or Na_2SeO_3 were injected into the yolk of broiler eggs to verify the results of the White Leghorn egg studies. Embryo livability at d 18 of incubation was significantly affected by dose. Livability ranged from embryo livability injected eggs ranged from 78-98 and 78-100% for Na_2SeO_3 and Se-Met treatments, respectively. Injecting eggs with 20 or 40 µg

Se/ egg resulted in decreased livability at both 18 and 20 d of incubation. However, no embryonic abnormalities were observed. Linear increases in all tissues were obtained with increasing doses of Se. Injecting broiler breeder eggs with Se as Se-Met or Na_2SeO_3 up to 40 μg Se/egg at day 10 of incubation has little effect on embryo viability and injecting Se-Met results in greater tissue Se accumulation than injecting the same amount of Se as Na_2SeO_3.

In the fourth study (Chapter 6), the same injection doses as Experiment 3 were used. In addition, hatched chicks were grown for 21 days. Mean body weights at 1, 7, 14 and 21 days of age were not affected by treatments. At 7 days of age, chicks from the Se-Met injection treatments showed higher lung, heart, but not liver, Se concentrations in contrast to those from the Na_2SeO_3 treatments. A significant elevation in tissue Se due to injecting Se-Met was also seen after 14 and 21 days in the lung, but not in other tissues. Induced lipid peroxidation observed in the lung and heart was significantly lower for Se-Met than for Na_2SeO_3. There was no difference seen in the liver except for the dose effect. Tissue homogenates from embryos that received the 0 μg Se had the highest concentration of TBARS, compared with other treatments. Immune response was measured following SRBC injection of chicks at 3 (primary) and 18 (secondary) days of age. Antibody titers were not significantly different at 7 days post primary and 4 days post secondary SRBC injection.

In the fifth study (Chapter 7), chicks were hatched from broiler breeder hens fed a corn-soybean meal diet without Se supplementation or with 0.3 ppm Se as either Na_2SeO_3 or Se yeast. The Se status, as measured by tissue Se concentrations of progeny chicks was improved at hatching by supplementing breeder hens with either forms of Se. However, the improvement was greater with Se yeast, compared with Na_2SeO_3.

The objective of this research was to study the effects of in ovo injection of graded levels of Se either as Se-Met or Na_2SeO_3 in fertile eggs after 10 d of incubation and determine the fate of Se once it has been absorbed and deposited into different embryonic tissues; its effects on embryo viability, lipid peroxidation, immune response and post hatch development. Previous studies on avian Se toxicity in the literaturee

reported that injecting Se in ovo resulted in high mortality and embryos with craniofacial and limb defects but this is not the case in the data presented in this dissertation. We cannot directly correlate Se injection with embryo lethality and abnormality since exposure to higher Se doses in our experiment did not result in such observations. The quality of solutions injected, the route and timing of injection as well as the quality of injection techniques can possibly explain the differences in the results gathered.

The results of these experiments indicate that in ovo injection of Se up to 60 μg Se/egg did not prove to be detrimental to the developing embryos enough to produce developmental defects. Injecting graded doses of Se as either Se-Met or Na_2SeO_3 resulted in linear increases in tissue Se concentration. However, increasing the dose of Na_2SeO_3, above 20 μg Se resulted in minimal increase in heart and breast se concentration, compared with Se-Met. The effects on tissue Se concentrations suggest that these compounds are metabolized differently by the chick embryo. Se-Met injection, compared with Na_2SeO_3, resulted in higher hatchability, reduced lipid peroxidation in the lung and heart muscle and Se concentrations in heart and breast muscle through 7 days and lung though 21 days of growth.

The results of these studies indicate that injection of Se into the yolk of incubating eggs may be useful for enhancing Se status during embryonic and early post-hatch development. Thus, the improvement in Se status using this method in conjunction with dietary Se supplementation of breeder hens would be much greater than with only using dietary supplementation.

APPENDIX 1: Flourometric Determination of Selenium

Reference:
Olson *et al.*, 1975 and Cantor and Tarino, 1982

Principle:

Samples undergo nitric/perchloric acid digestion, titration, reaction with 2,3 diaminonaplthalene and extraction with cyclohexane. Cyclohexane layer is used to read relative fluorescence Using regression procedures, slenium conncetration in (micrograms) is determined.

Apparatus:

Analytical balance (readability to 0.0001g)
Perchloric hood
Glass Culture tubes with caps (200 x 25 mm)
Turner Fluorometer Model 450 equipped with excitation filter #NB520 and emission filter #NB360 and apperture slides 1X,3X,10X and 30X
Boiling water bath
50C water bath

Reagents:

Nitric acid – certified ACS plus grade
Perchloric acid 70%- reagent grade
Ammonium hydroxide – certified ACs grade
Hydrochloric acid – trace mineral grade
1:4 concentration
1:9 concentration
0.1N
Hydroxylamine-EDTA solution
In 1000 ml volumetric flask, dissolve 9 g disodium ethylene tetracetate in 900 ml water. Add 25 mg hydroxylamine hydrochloride, mix until dissolved. Dilute to 1 L with water.
Cresol Red
Dissolve 0.05g cresol red in 1 ml water and add 1 drop ammonium hydroxide. Add 249 ml water; mix until dissolved.
2,3 diaminonapthalene (DAN) solution
Concentration needed is 1 mg/100 ml of 0.1N HCl. 5 ml is needed for each sample, standard and blank. Use 99% purity DAN. Prepare in amber bottle mix slowly with magnetic stirrer. Extract 3x with cyclohexane in separatory funnel prior to use; discard the cyclohexane layer.
Note: DAN is light sensitive. When weighing DAN,turn off fluorescent light, yellow light may be used.

Procedures

Sample preparation

1. Weigh approximately 1 g of homogeneous sample into the culture tubes. Prepare samples in duplicate.
2. Add about 3 glass beads to each sample.
3. Add 15 ml nitric acid to tissue samples.
4. Add 2.5 ml perchloric acid.
5. For the nitric acid blanks (prepare three), add 15 ml nitric acid and 2.5 ml perchloric acid to the culture tube.
6. Cover with plastic wrap. Let stand overnight in fumehood.

Standard preparation

Standards do not undergo digestion and do not contain nitric acid.

1. Prepare a selenium standard in triplicate. Curve should cover expected sample range. For the 0, use 1 ml of water and 2.5 ml perchloric acid.

Digestion

Fumehood with capacity to accommodate nitric acid and perchloric acid must be used in the procedure.

1. Heat on Labconco digestors on low heat until deep orange (nitric acid) fumes disappear.
2. Increase heat when samples becomes transparent.
3. The nitric acid is gone when sample volume reduses and clears. Perchloric fumes are dense and white and recirculate at the base of the flask. Once the perchloric fumes appear, heat sample 30 minutes to ensure nitric acid is completely gone.
4. Cool sample in fumehood until fuming ceases.

Boiling to titration

1. Add 2.5 ml 1:9 HCl to all samples., standards and blanks; vortex.
2. Heat uncoverd samples in boiling water bath for 25 minutes.
3. Cool completely and add 100 ul of cresol red to each sample.
4. In fumehood, add ammonium hydroxide dropwise to yellow point. Rinse inside of flask/tube with water.
5. Add 1:4 HCl dropwise to orange endpoint (pH 1.5)

DAN reaction

1. Add 5 ml DAN solution to each sample.
2. Cover sample tubes with plastic wrap and heat in 50C water bath for 25 minutes.
3. Remove from water bath and place in cool water bath. Cool completely.
4. Add 8 ml cyclohexane.
5. Tighly cap tubes and mix by slowly inverting covered racks for 1 minute.
6. Bring cyclohexane level up in the tubes by adding 0.1N HCl.
7. Using disposable tranfer pipet, transfer cyclohexane layer to borosilicate glass tubes, 12 x 75 mm.

Fluorometer

1. Install NB360 filter for excitation and NB520 fro emission wavelengths and aperture slides.
2. Adjust to zero using black rod. Adjust to zero each time aperture slides are changes.
3. Wipe each tube with Chemwipe. Read the standard curve, samples and blanks.

Se determination

Using regression procedures, generate the regression equation and calculate selenium concentration of samples.

APPENDIX 2: Protocol for Lipid Peroxidation of Liver Whole Cell Fractions Using Ascorbic Acid Stimulation

Reference:

modified from Noguchi T, Cantor AH, Scott ML. (1973). Mode of action of selenium and vitamin E in prevention of exudative diathesis in chicks. J Nutr. 103(10):1502-11

Principle:

Samples are incubated with ascorbic acid to stimulate lipid peroxidation, presence of glutathione peroxidase inhibit the formation of lipid peroxides. Oxidation is measured by the formation of thiobarbituric acid reactive substances *i.e.*, malonaldehyde.

Apparatus:

Tekmar® Homogenizer
Mistral® 2000, refrigerated centrifuge
Water bath
Hot plate
Culture tubes (12 x 75 mm)
Beaker (4L)
Volumetric flask (2L)
Digital thermometer
Wire baskets/ wire racks
Pipets
Pipette tips
Filter paper # 541

Reagents:

0.025M Tris-HCl (157.6 g/mole)
Weigh 7.88 g Tris-HCl and dissolve in 1700 ml deionized water. Adjust pH to 7.4. Transfer to 2 L volumetric flask and dilute to 2 L.

0.174M KCl (74.56 g/mole)
Dissolve 12.97 g KCl in 1L 0.025M Tris-HCl buffer. Adjust pH to 7.4

0.67% Thiobarbituric acid (TBA)
Dissolve 0.67g TBA in 100 ml deionized water. Do not refrigerate. Store in amber bottle with stirring bar.

20% Trichloroacetic acid (TCA)
Dissolve 20 g TCA in 100 ml deionized water

1.5mM ascorbic acid (176.13 g/mole)in Tris-KCL buffer
Dissolve 0.0528 g ascorbic acid in 200 ml Tris-KCl buffer

Procedure:

*Sample Preparation**

1. Homogenize 2g of tissue sample (liver, lung, heart, breast muscle) in 20ml 0.174M KCl in 0.025M Tris-HCl buffer (buffer pH7.4) using a Tekmar® homogenizer.
2. Centrifuge homogenate at 400 X g for 10 mins. (Mistral® 2000 refrigerated centrifuge)
3. Transfer supernatant into a new 20ml conical tube and discard the precipitate. *Note: samples and reagent should be on ice while performing the assay*

Lipid Peroxidation Stimulation Using Ascorbic Acid

1. For time 60 samples, mix 0.5ml of the whole cell fraction in 1ml of 1.5mM ascorbic acid solution in Tris-KCl buffer + 1.5ml Tris-KCl buffer (use a 12 x 75 mm borosilicate tube)
2. For time 0 samples, pipet 0.5ml of the whole cell fraction and add 1ml of 20% TCA, vortex. Then add 1ml of 1.5mM ascorbic acid solution in Tris-KCl buffer + 1.5ml Tris-KCl buffer
3. Incubate samples at 37C for 1 hour in a water bath.
4. Stop the reaction by adding 1ml of 20% (w/v) TCA in the time 60 samples.

TBA (thiobarbituric acid) Lipid Peroxide Formation Test

1. Filter samples using a whatman #541 filter paper (use the 16 X 125 mm borosilicate tubes)
2. Transfer 2 ml of filtrate in a fresh clean 16 X 125 mm tubes then add 1 ml of 0.67% TBA,
3. Boil samples (100C) for 35 mins.
4. Cool completely in water for 5 mins
5. Read absorption at 530 mu

APPENDIX 3: Antibody Response to Sheep Red Blood Cell (SRBC)

Reference:

Gore, A.B. and M. A. Qureshi 1997. Poult. Sci. 76:984-991.
Yamamoto, Y., and B. Glick. 1982. Poult. Sci. 61:2129-2132.
Grimes S.E. 2002. ISBN 974-7946-26-2.

Principle:

At 3 d of age, birds fro each treatment groups will be injected, intravenously with 1 ml of 7% SRBC, collect blood prior to inoculation. Blood collection will be at 7 and 14 d post primary injection. At 14 d post primary challenge, all birds will be challenged with a secondary SRBC injection; blood will be collection will be at 4 d post-challenge. Anti-SRBC injection titer will be calculated using micro hemagglutination and titers are expressed as $\log_2$ of the reciprocal of the highest dilution showing visible agglutination.

Apparatus

Microtiter Plate: "U" bottom, sterile
Pipette tips 1-200ul capacity
Multichannel pipettor
Reservoir
Water bath

Reagents:

Washed Sheep Red Blood Cell (100%)
2-Mercaptoethanol (ME)
To 0.7 ml ME, add 99.3 ml deionized water
Sterile PBS
Dissolve 2 PBS tablet in 250 ml deionized water.

Procedure:

Note: Heat inactivate samples at 56°C one day prior to analysis.

For Total plate (PBS)

1. Add 50 µl of PBS in the first row of the wells of the microtiter plate.
2. Add 50 µl of serum sample in each well with PBS.
3. Cover plates and incubate for 30 mins. at 37°*C*.
4. Remove plates from incubator and add 50 µl PBS in the remaining well.
5. Dilute samples, using multichannel pipetor, aspirate 50 µl from the first well and transfer into the second well.

6. Mix 4-5x by pipetting up and down and then transfer 50 µl to the next well and do the same till the last well, discard the last 50 µl.
7. Add 50 µl of 2% SRBC to all the wells.
8. Cover and incubate for 30 mins at 37°*C*.
9. Read immediately.

For ME-titer (PBS/Mercaptoethanol)

1. Add 50 µl on 0.01 ml merccaptoethanol in PBS in the first row of the wells of the microtiter plate.
2. Add 50µl of serum sample in each well with ME/PBS.
3. Cover plate and and incubate for 30 mins. at 37°*C*.
4. Remove plates from incubator and add 50 µl PBS in the remaining well.
5. Dilute samples, using multichannel pipettor, aspirate 50 µl from the first well and transfer into the second well.
6. Mix 4-5x by pipetting up and down and then transfer 50 µl to the next well and do the same till the last well, discard the final 50 µl.
7. Add 50 µl of 2% SRBC to all the wells.
8. Cover and incubate for 30 mins at 37°*C*.
9. Read immediately.

Titers are read and recorded as ME-resistant (IgG). The ME-sensitive (IgM) is the difference between the PBS (Total) and ME-R readings. Total-ME-R=ME-S

Note: Photograph of positive and negative titer readings can be found at www. fao.org/docrep/005/ac802e/ac802e0a.html

APPENDIX 4: Blood Collection Through Venipuncture

References:

www. fao.org/docrep/005/ac802e/ac802e0a.html

Principle:

1. To obtain serum which will be tested SRBC antibodies, no anticoagulant is required and the blood is allowed to clot. The levels of antibody detected in individual birds give an indication of the response to SRBC challenge.

Wing vein bleeding

Materials

- 2.5 mL syringes
- 25 gauge needles for small chickens
- 23 gauge needles for larger chickens
- Cotton wool
- 70 percent alcohol solution
- Labels or marking pen to label each syringe

Method

1. Hold the chicken horizontally on its back. Hold the legs and place the other hand under the back to support the chicken. (Assistant)

2. Pull a wing of the chicken out towards you. (Bleeder)

3. Note the wing vein, clearly visible running between the biceps and the triceps muscles. The wing vein forms a V (bifurcates). Note the tendon of the pronator muscle that runs across the V.

4. Pluck away any small feathers that obscure the vein.

5. Disinfect the area around the bleeding site by swabbing with 70 percent alcohol.

6. Insert the needle under the tendon. Direct the needle into the wing vein in the direction of the flow of blood. Do not insert the needle too deeply. Keep clear of the ulnar nerve.

7. Once the tip of the needle is in the vein, gently pull the plunger of the syringe. Blood will flow into the syringe. If blood does not flow, release the plunger and make a very

slight adjustment to reposition the end of the needle.

8. Use a gentle suction to withdraw the blood to prevent the veins from collapsing.

9. After removing the needle, apply pressure to the vein for a few seconds to discourage further bleeding.

10. Transfer the collected blood into a vaccutainer and place it an angle in a rack facilitate clotting

Note: Chicken wing vein collection photograph can be viewed at
www. fao.org/docrep/005/ac802e/ac802e0b.jpg

Blood collection via the jugular vein

1. Hold the chicken horizontally on its back. Hold the legs and place the other hand under the back to support the chicken.(Assistant)
2. Stretch out the neck with one hand away from you, remove some feathers along the neck area. The jugular vein is now visible. (Bleeder)
3. Place the needle at an angle, bevel up, against the vein.
4. Insert the needle through the skin. Direct the needle into the jugular vein in the direction of the flow of blood. Do not insert the needle too deeply. Keep clear of the trachea.
5. Puncture the vein and slowly withdraw blood.
6. Use a gentle suction to withdraw the blood to prevent the veins from collapsing.
7. After removing the needle, apply pressure to the vein for a few seconds to discourage further bleeding.

8. Transfer the collected blood into a vaccutainer and place it an angle in a rack facilitate clotting

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VITA

LIZZA M. MACALINTAL

Place of birth: Philippines

Date of Birth: July 2**,** 1968

Education

MASTER OF SCIENCE, 2004

North Carolina State University, Raleigh, NC

DOCTOR OF VETERINARY MEDICINE, 1992

University of the Philippines at Los Banos, Philippines

Scholastic and Professional Awards:

Student Award for Excellence (Metabolism and Nutrition)

2011 Poultry Science Association, St. Louis, MO

Fulbright Scholarship

2002-2004 (NC State University)

Professional Work Experience:

Veterinarian – Bureau of Animal Industry, Philippines, 2000-2006

Lizza M. Macalintal

April 26, 2012

Date