Minerals and anti-oxidants

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Introduction

Animal health depends on many factors and it is increasingly appreciated that diet plays a pivotal role in maintaining health and preventing disease. Among many dietary factors, minerals have special importance in the maintenance of fast growth, reproduction and immuno-competence in poultry production. This concept is based on understanding the contribution of minerals in reducing the detrimental effects of free radicals and toxic metabolites on immune processes in the animal's body.

Natural mineral anti-oxidants in feed ingredients

The anti-oxidant/pro-oxidant balance can be modulated by sub-optimal diets and poor nutrient intakes, or positively affected by dietary supplementation. Therefore, feed components can change this balance and may influence such effects as the rate of ageing and disease resistance in human and animals. The most important step in balancing oxidative damage and anti-oxidant defence in the animal's body is enhancing anti-oxidant capacity by optimising the dietary intake of anti-oxidant compounds.

Animal feeds contain a range of different compounds that possess anti-oxidant activities, many of them being minerals or mineral–dependent. The key minerals in animal feed are listed below, and many of these are involved in anti-oxidation (Surai, 2002; Surai and Dvorska, 2002).

Selenium

- trace element
- essential part of a range of selenoproteins, including glutathione peroxidase (GSH-Px), thioredoxin reductase (TrxR), iodothironine

deiodinase (ID) and some others. In the animal and human body 25 selenoproteins have been identified to date

- food ingredients contain variable concentrations of Se, but most of them are deficient in this element
- physiological requirement is low, but if not met, anti-oxidant system is compromised with detrimental consequences for animal health
- toxic in high doses
- there are two major sources of Se for animals: a natural source in the form of various selenoamino acids including selenomethionine and selenocysteine or inorganic selenium in the form of selenite or selenate
- organic selenium supplementation has physiological and biochemical benefits for animals, including poultry.

Zinc

This is the second most abundant trace element in mammals and birds and is a component of over 300 enzymes participating in their structure or in their catalytic and regulatory actions in most species. It takes part in:

- anti-oxidant defence as an integral part of SOD
- hormone secretion and function (somatomedin-c, osteocalcin, testosterone, thyroid hormones, insulin, growth hormone)
- keratin generation and epithelial tissue integrity
- bone metabolism being an essential component of the calcified matrix
- nucleic acid synthesis and cell division
- protein synthesis
- catalytic, structural and regulatory ion for enzymes, proteins and transcription factors and participates in the metabolism of carbohydrates, lipids and proteins
- sexual development and spermatogenesis
- immune function
- appetite control via acting on the central nervous system

Organic Zn has higher availability in comparison to inorganic sources and is considered to be more beneficial for animal health.

Copper

Copper is an essential component of a range of physiologically important metalloenzymes and takes part in:

- anti-oxidant defence as an integral part of SOD
- cellular respiration
- cardiac function
- bone formation
- carbohydrate and lipid metabolism
- immune function
- connective tissue development
- tissue keratinization
- myelination of the spinal cord

The main Cu-containing enzymes are shown in Table 1. Inorganic copper has a strong pro-oxidant effect and (if not bound to proteins) can stimulate lipid peroxidation in feed or the intestinal tract (Surai et al., 2003a). Organic copper does not possess pro-oxidant properties and can improve the copper status of animals.

Table 1. Copper-containing enzymes in animals and humans (Adapted from Nath, 1997).

Common name	*EC number*	*Functional role*	*Known or possible consequence of deficiency*
Cytochrome c oxidase	1.9.3.1	Electron-transport chain	Muscle weakness; cardiomyopathy, brain degeneration
Superoxide dismutase	1.15.1.1	Free radical detoxification	Membrane damage; other free radical damage
Thyrosinase (monophenol monooxygenase)	1.14.18.1	Melanin production	Failure of pigmentation
Dopamine -ß-hydroxylase	1.14.17.1	Catecholamine production	Neurological effects
Lysyl oxidase	1.4.3.13	Cross-linking of collagen and elastin	Vascular rapture; loose skin and joints; osteoporosis
Ceruloplasmin	1.16.3.1	Ferroxidase, anime oxidase; Cu transport	Anaemia; deficient supply of Cu to other tissues
Clotting factor V		Blood clotting	Bleeding tendency

Iron

Iron has a vital role in many biochemical reactions taking part in:

- anti-oxidant defence as an essential component of catalase
- energy and protein metabolism
- heme respiratory carrier
- oxidation/reduction reactions
- electron transport system

Iron is a very strong pro-oxidant and if not bound to proteins can stimulate lipid peroxidation. This is especially relevant to the digestive tract where lipid peroxidation can be stimulated, causing enterocyte damages and decreased absorption of nutrients (Surai et al., 2003a). If iron is included in the premix in inorganic form, it can stimulate vitamin oxidation during storage. Therefore organic iron is a solution to avoid these problems and improve the iron status of animals.

Manganese

Manganese plays an important role in body metabolism as an essential part of a range of enzymes taking part in:

- anti-oxidant protection as an integral part of SOD
- bone growth and egg shell formation
- carbohydrate and lipid metabolism
- immune and nervous function
- reproduction

As with other organic minerals, manganese seems to be better assimilated from the diet

From this brief list of mineral functions, it can be clearly seen that most are relevant to immune function, via involvement in the anti-oxidation processes.

The need for anti-oxidant defence

Free radicals are atoms or molecules containing one or more unpaired electrons. Most biologically relevant free radicals are derived from oxygen and nitrogen, the so-called reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both these elements are essential, but in certain circumstances are converted

into free radicals. These are highly unstable, and their reactive capacity makes them capable of damaging biologically relevant molecules such as DNA, proteins, lipids or carbohydrates (Surai, 2002).

The animal's body is under constant attack from free radicals, formed as a natural consequence of the body's metabolic activity and the immune system's strategy for destroying invading micro-organisms (Table 2). For example, under normal physiological conditions about 3-5% of the oxygen taken up by the cell undergoes univalent reduction leading to the formation of free radicals (Singal et al., 1998). About 10^{12} O_2 molecules are processed by each rat cell daily with 2% leakage from cells, yielding a total of 2 $x10^{10}$ molecules of ROS per cell per day (Chance et al., 1979). Furthermore Helbock et al. (1998) have calculated that the DNA in each cell in a rat is hit by about 100,000 free radicals a day and each cell sustains as many as 10,000 potentially mutagenic (if unrepaired) lesions per day arising from endogenous sources of DNA damage (Ames and Gold, 1997). If oxidative lesions are not repaired and exposure increases with age, then an old rat can accumulate approximately 66,000 oxidative DNA lesions per cell (Ames, 2003). An interesting calculation has been made by Halliwell (1994) where it was assumed that in mitochondria about 1-3% of oxygen consumed might leak from the electron transport chain forming superoxide radicals. They calculated that an adult at rest utilises approximately 3.5 ml O_2 per kg body weight per minute or 352.8 litres per day (assuming 70 kg body mass) or 14.7 moles per day. Therefore if 1% of the oxygen becomes superoxide this equate to 0.147 moles per day or 53.66 moles per year or about 1.72 kg per year of superoxide radicals. This represents a substantial increase in physiological stress levels.

Table 2. Selected sources of free radicals (Adapted from Surai, 2002)

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

Such calculations demonstrate the substantial free radical production in the body and the great potential for damage to tissues and cells. The internal and external sources of free radicals are

shown in Table 2. It is interesting that free radicals also work as physiological mediators and signalling molecules; therefore complete removal of free radicals from the cell could have detrimental consequences.

In the case of the immune system, the problem is exacerbated as immune cells produce free radicals and use them to destroy pathogens (Surai, 2002). High oxygen concentration is potentially toxic for living organisms, as first described in laboratory animals in 1878 (Knight, 1998).

Living organisms have evolved specific anti-oxidant protective mechanisms to deal with the free radicals constantly produced by cells. These mechanisms helped organisms to survive in an atmosphere when oxygen concentration was increasing millennia ago, and are described by the general term "anti-oxidant system" (Halliwell and Gutteridge, 1999; Surai, 2002). In nature there are thousands of compounds possessing anti-oxidant properties that are able to neutralise free radicals. They can be fat-soluble (vitamin E and carotenoids, coenzyme Q, etc.) and water-soluble (ascorbic acid, glutathione, bilirubin, taurine, etc.), or synthesised in the body (ascorbic acid, glutathione or taurine) or have to be delivered via feed (minerals, vitamin E, carotenoids).

There is a range of mineral-dependent anti-oxidant enzymes, which can be synthesised in the body and are able to effectively deal with free radicals, but require feed-derived mineral co-factors to do so. For example, Se in the form of selenocysteine is an essential part of a family of enzymes called glutathione peroxidases (GSH-Px) and thioredoxin reductases (TR) Zn, Cu and Mn are integral parts of another anti-oxidant enzyme family called superoxide dismutases (SOD) and iron is an essential part of the anti-oxidant enzyme called catalase. When these metals are delivered in feed in sufficient amounts the body can synthesise anti-oxidant enzymes. In contrast deficiency of these elements causes oxidative stress and damages to biological molecules and membranes.

How anti-oxidants work

Biological anti-oxidants react with free radicals or precursor metabolites converting them into less reactive molecules and preventing or delaying oxidation of biological molecules. The most important and well-characterised natural anti-oxidants in the animal's body are vitamins E and C. Plant pigments such as carotenoids have anti-oxidant capacity. Protective anti-oxidant compounds are located in organelles, subcellular compartments or the extracellular space enabling maximum cellular protection to occur.

In fact all anti-oxidants in the body are working together to achieve physiological defence. The anti-oxidant 'team' acts to control levels of free radical formation, as a coordinated system where deficiencies in one component impacts the efficiency of others. For example, ascorbate can help vitamin E to recover from oxidation to become active again. If relationships in this team are effective, where the individual has a balanced diet and sufficient intake of anti-oxidants, then even low doses of such anti-oxidants as vitamin E or Selenium can be effective. When the anti-oxidant system finds itself in high stress conditions, if free radical production is increased dramatically, without external help it will be difficult to prevent damage to organs and cells. Such external help can be provided by dietary supplementation with increased doses of natural anti-oxidants, especially minerals such as selenium. For nutritionists or feed formulators the challenge is to understand when the anti-oxidant team requires help and how much of this help can justify extra feed expense, because anti-oxidants are typically expensive components of the diet. A list of possible stresses in poultry production includes the following (Surai, 2002):

- *Time* between an egg being laid and it's cooling down for storage. Eggs should be collected frequently in hotter climates. In such conditions free radical damages to lipids and proteins can occur and anti-oxidant protection is beneficial.
- *Egg storage before incubation* often associated with lipid peroxidation within egg membranes, particularly those containing high levels of PUFAs. Increased Se concentration in combination with other anti-oxidants (vitamin E and carotenoids) can be an effective means to prevent damaging effects of free radicals produced within the egg.
- *Temperature, humidity and carbon dioxide concentration* fluctuations during incubation can affect embryonic development, oxidation and phosphorylation in tissues leading to free radical production. For example, high carbon dioxide concentrations during the incubation period has been shown to jeopardise the liveability of the embryo
- *Day 19 of embryonic development* is an important point when risk of lipid peroxidation is very high. At this stage chick tissues are characterised by comparatively high levels of polyunsaturated fatty acids (PUFA). At the same time natural anti-oxidant reserves have not reached a sufficient level for innate protection. At this stage of development 'piping' occurs; and oxygen availability for tissues increases. Low anti-oxidant status in combination with high temperature, humidity, and PUFAs can increase susceptibility to lipid peroxidation.

- *Hatching time* is considered as an environmental stress for the chick. At this point natural anti-oxidant concentrations have reached a maximum, but high levels of lipid unsaturation in tissues (decreasing concentration of ascorbic acid) can limit vitamin E recycling and high temperature and humidity increase risk of lipid peroxidation.
- *Delay in collecting chicks from incubator*. Since not all chicks are hatched at the same time (eggs from older breeders hatch earlier than those from young flocks and chicks from smaller eggs hatch earlier than those from large eggs), some may be in the incubator for 2-12 hours longer than others. This puts pressure on anti-oxidant defence capacity. Furthermore, any delay in food and/or water intake after hatching usually negatively affect a number of performance parameters, and delays the maturation of the enzymatic systems that control metabolism, free radical production and anti-oxidant protection systems.
- *Transportation from hatchery to farm* is another source of stress. For breeding companies where chicken transportation can involve several thousand miles, a very high degree of stress should be associated with temperature fluctuation and dehydration.
- *Sub-optimal temperatures in the poultry house*. Cold tolerance as well as feather cover is influenced by thyroid hormone activity, which is Se-dependent.
- *High levels of ammonia and CO_2 in poultry house as a result of inadequate ventilation* can substantially decrease anti-oxidant system efficiency.
- *Disease challenge*. Phagocytic immune cells produce free radicals in the process of killing internalised pathogens. Without adequate anti-oxidant nutrient reserves, cellular membranes and important organelles, can be damaged by the free radicals thereby reducing the effectiveness of the immune cell. In addition, Se is considered to have a specific role in immune system regulation, which is independent of its anti-oxidant functions.
- *Vaccination* is a substantial stress; and in some cases using vitamin E as a vaccine adjuvant can help improve vaccination efficiency.
- *Induced moulting with feed withdrawal* is an important stress condition where decreased efficiency of heterophil function increases bird susceptibility to various infections.
- *Mycotoxins in the feed* can decrease anti-oxidant assimilation

from the feed and increase their requirement to prevent damaging effects of free radicals and toxic products of mycotoxin metabolites. It is now recognised that at least 25% of world's grains are contaminated with mycotoxins.

- *Heavy metals and other toxins (dioxin, pesticides, fungicides, herbicides, etc.) in the feed* can also cause an oxidative stress, decreasing immunocompetence, productive and reproductive performances and increasing a requirement for anti-oxidants.
- *Oxidized fat in the diet* increases the exposure to free radicals, inducing stress in the intestine and increasing anti-oxidant requirement to prevent damage to tissues. When a chicken diet includes fat which has undergone high temperature treatment, resulting peroxides can contribute substantially to oxidative stress.
- *Extensive preventive medication (coccidiostats or other veterinary drugs in the diet)* can limit dietary anti-oxidant uptake or increase stress conditions, e.g. monensin can stimulate lipid peroxidation in the chicken liver. Similarly, oral furazolidone treatment of chickens has been associated with a decreased vitamin E concentration and increased lipid peroxidation in their liver.
- *Vitamin A excess* in the diet has been shown to cause oxidative stress, decreasing vitamin E and carotenoid concentrations in tissues and increasing tissue susceptibility to lipid peroxidation.

The list of potential stresses can vary from one poultry farm to another, but overproduction of free radicals and the critical need for anti-oxidant protection are common factors.

Three levels of anti-oxidant defence

There are certain anti-oxidant considerations for adequate defence that the nutritionist must take into account when deciding the level of exposure the animal faces. Protective anti-oxidant compounds are located in organelles, subcellular compartments or in extra-cellular space, enabling maximum protection. The anti-oxidant system of the living cell includes three major levels of defence (Niki, 1996; Surai, 1999; Surai, 2002):

- The first level of defence is responsible for prevention of free radical formation by removing precursors of free radicals or by inactivating catalysts and consists of three anti-oxidant enzymes namely SOD, GSH-Px and CAT plus metal-binding proteins (Figure 1).

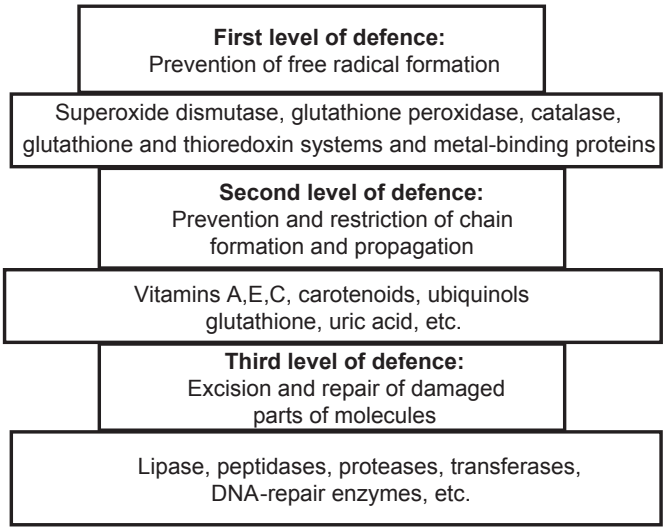

Figure 1. Three levels of antioxidant defence in animal cells (Adapted from Surai, 2002)

- Since the superoxide radical is the main free radical produced under physiological conditions in the cell (Halliwell, 1994) the enzyme superoxide dismutase (EC 1.15.1.1) is considered to be the main element of the first level of anti-oxidant defense in the cell (Surai, 1999). This enzyme transforms the superoxide radical in the following reaction:

$$2O_2^{*} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide dismutase was discovered by McCord and Fridovich in 1969 who discovered the enzymatic activity in preparations of carbonic anhydrase or myoglobin that inhibited the aerobic reduction of cytochrome C by xanthine oxidase. This discovery opened up a new era in free radical research. At present, three distinct isoforms of SOD have been identified in mammals, and their structure and protein content have been described (Zelko *et al*., 2002). SOD1, or Cu-Zn-SOD (previously called haemocuprein, Bannister, 1988), was the first enzyme of this family to be characterised and is a copper and zinc-containing compound that is found almost exclusively in intracellular cytoplasmic spaces. It exists as a 32 kDa homodimer and is present in the cytoplasm and nucleus of every cell type examined (Zelko *et al*., 2002).

The second member of the family has manganese (Mn) as a cofactor and is called Mn-SOD. It was shown to be a 96 kDa homotetramer, located exclusively in the mitochondrial matrix, a prime site of superoxide radical production (Halliwell and Gutteridge, 1999).

Therefore the expression of Mn-SOD is considered to be essential for the survival of aerobic life and the development of cellular resistance to oxygen radical-mediated toxicity (Fridovich, 1995). Mn-SOD is an inducible enzyme and its activity is affected by cytokines and oxidative stress. In fact, Mn-SOD has been shown to play a major role in promoting cellular differentiation and in protecting against hyperoxia-induced pulmonary toxicity (Fridovich, 1995). In 1982 a third SOD isozyme was discovered by Marklund and co-workers and called extracellular superoxide dismutase (EC-SOD), due to its exclusive extracellular location. EC-SOD is a glycoprotein with a molecular weight of 135,000 kDa with high affinity for heparin. However, there are some species-specific variations in molecular weight. EC-SOD is present in various organisms as a tetramer or, less commonly, as a dimer and contains one copper and one zinc atom per subunit, which are required for enzymatic activity (Fattman *et al*., 2003). The expression pattern of EC-SOD is highly restricted to the specific cell type and tissues where its activity can exceed that of Cu-Zn-SOD or Mn- SOD. The fourth form of the enzyme Fe-SOD was isolated from various bacteria but is not found in animal tissues (Michalski, 1992). Furthermore, a novel type of nickel-containing SOD was purified from the cytosolic fractions of *Streptomyces* species (Youn *et al*., 1996). The biosynthesis of SODs, in most biological systems, is well controlled. In fact, exposure to increased oxygen levels, increased intracellular fluxes of O_2- and metal ions perturbation, and exposure to environmental oxidants appear to influence the rate of SOD synthesis in both prokaryotic and eukaryotic organisms (Hassan, 1988).

The hydrogen peroxide formed by SOD action can be detoxified by GSH-Px or CAT which reduce it to water as follows:

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} GSSG + 2H_2O$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Catalase (CAT) (EC 1.11.1.6) is a tetrameric iron dependant enzyme consisting of four identical subunits of 60 kDa containing a single ferriprotoporphyrin group per subunit. It plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Mates *et al*., 1999). In mammalian cells, NADPH is bound to catalase protecting it from inactivation by H_2O_2 (Chaudiere and Ferrari-Illiou, 1999). Since GSH-Px has a much higher affinity for peroxide than CAT (Jones et al., 1981) and wider distribution in the cell (catalase is located mainly in peroxisomes), peroxide removal from the cell is very much dependent on GSH-Px. Recently it has been shown that thioredoxin peroxidases are

also capable of directly reducing hydrogen peroxide (Nordberg and Arner, 2001). It is interesting that the levels of anti-oxidant enzymes are regulated by gene expression as well as by post-translational modifications (Fujii and Taniguchi, 1999).
Transition metal ions accelerate the decomposition of lipid hydroperoxides into cell-toxic products such as aldehydes, alkoxyl radicals and peroxyl radicals:

$$LOOH + Fe^{2+} \longrightarrow LO^{*} + Fe^{3+} + OH^{-}$$
$$LOOH + Fe^{3+} \longrightarrow LOO^{*} + Fe^{2+} + H^{+}$$

Therefore, metal-binding proteins (transferrin, lactoferrin, haptoglobin, hemopexin, metallothionenin, ceruloplasmin, ferritin, albumin, myoglobin, etc.) also belong to the first level of defence. It is necessary to take into account that inorganic and charged iron and copper compounds are powerful promoters of free radical reactions and their availability in "catalytic" forms must be carefully regulated *in vivo* (Halliwell, 1999). To ensure this, organisms have evolved to keep transition metal ions safely sequestered in storage or transport proteins. In this way the metal-binding proteins prevent formation of hydroxyl ions by preventing them from participation in radical reactions. For example, transferrin binds iron (about 0.1% of the total body reserves), transports it within the plasma pool and attaches it to the transferrin receptor, where it will not be able to catalyse free radical reactions. Ferritin is considered to be involved in iron storage (about 30% of total body reserves) within the cytosol in various tissues including liver and spleen.

The majority of iron in the body (55-60%) is associated with hemoglobin within red cells and about 10% with myoglobin in muscles (Galey, 1997). A range of other iron-containing proteins (mainly enzymes) can be found in the body including NADH dehydrogenase, cytochrome P450, ribonucleotide reductase, proline hydroxylase, tyrosine hydroxylase, peroxidases, catalase, cyclooxygenase, aconitase, succinate dehydrogenase, etc. (Galey, 1997). Despite the importance of iron in numerous biochemical reactions, iron can be dangerous when not carefully managed via proteins. In fact, under certain stress conditions, a release of free iron from the normal storage sites can occur, whereby the superoxide radical can release iron from ferritin and H_2O_2 degrades the heme of hemoglobin to liberate iron (Halliwell, 1987). Recently it has been suggested that pro-oxidant properties of inorganic iron could have detrimental consequences for the digestive tract (Surai et al., 2003). Therefore, organic iron should be the nutritionist's choice for dietary supplementation.

Ceruloplasmin is a copper-binding protein that mediates free radical metabolism. Under physiological conditions it binds six or seven

copper ions per molecule, preventing their participation in free radical generation. About 5% of human plasma copper is bound to albumin or amino acids, the rest being bound to ceruloplasmin. Furthermore ceruloplasmin possesses anti-oxidant properties, being able to scavenge superoxide radical (Yu, 1994). It is now quite clear that metal sequestration is an important part of extracellular anti-oxidant defence. Similar to inorganic iron, inorganic copper is also a strong pro-oxidant in the digestive tract (Surai et al., 2003) and therefore organic copper is a preferential form of dietary supply.

Unfortunately this first level of anti-oxidant defence in the cell is not sufficient to completely prevent free radical formation and some radicals do escape through the preventive first level of anti-oxidant safety screen initiating lipid peroxidation and causing damage to DNA and proteins. Therefore the second level of defence consists of chain-breaking anti-oxidants - vitamin E, ubiquinol, carotenoids, vitamin A, ascorbic acid, uric acid and some other anti-oxidants. Glutathione and thioredoxin systems play a substantial role in the second level of anti-oxidant defence (Surai, 2002). Chain-breaking anti-oxidants inhibit peroxidation by reducing the propagation reaction as much as possible, limiting the potential cascade reaction and proliferation of free radicals. They prevent the propagation step of lipid peroxidation by scavenging peroxyl radical intermediates in the chain reaction:

$$LOO^* + Toc \longrightarrow Toc^* + LOOH$$

(LOO* is lipid peroxyl radical; Toc - tocopherol, Toc* - tocopheroxyl radical, LOOH – lipid hydroperoxide).

Vitamin E, the most effective natural free radical scavenger identified to date, is the main chain breaking anti-oxidant in the cell. However, hydroperoxides are produced in the reaction of vitamin E with the peroxyl radical and are toxic and if not removed as they impair membrane structure and functions (Gutteridge and Halliwell, 1990). In fact, lipid hydroperoxides are not stable and in the presence of charged transition metal ions can decompose producing new free radicals and cytotoxic aldehydes (Diplock, 1994). Therefore hydroperoxides have to be removed from the cell in the same way as H_2O_2, but catalase is not able to detoxify these compounds and only Se-dependent GSH-Px can deal with them converting hydroperoxides into non-reactive products (Brigelius-Flohe, 1999) as follows:

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} ROH\ (\text{non-toxic}) + H_2O + GSSG$$

Thus, vitamin E performs only half the job in preventing lipid

peroxidation by scavenging free radicals and forming hydroperoxides. The second part of this important process of anti-oxidant defence is due to Se-GSH-Px. It is necessary to underline, that vitamin E and selenium work in a tandem; and even very high doses of dietary vitamin E cannot replace Se which is needed (in the form of GSH-Px and thioredoxin reductase) to complete the second part of anti-oxidant defence as mentioned above. Thus, Se (as an integral part of the GSH-Px and thioredoxin reductase) belongs to the first and second levels of anti-oxidant defence. Indeed, anti-oxidant interactions and recycling is a key mechanism of the effective anti-oxidant defence (Figure 2).

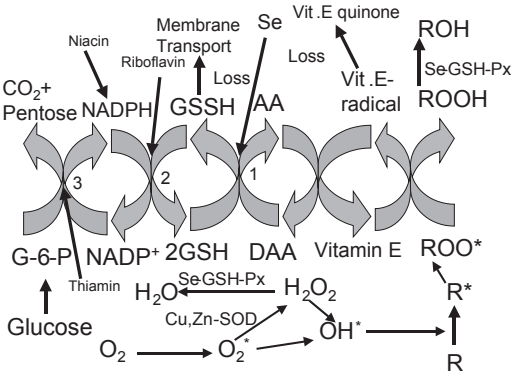

Figure 2. Radical formation and antioxidant defence (Adapted from Surai, 2002)

As a result of anti-oxidant action of vitamin E the tocopheroxyl radical is formed. This radical can be reduced back to an active form of a-tocopherol by coupling with ascorbic acid oxidation. Ascorbic acid can be regenerated back from the oxidised form by recycling with glutathione which can receive a reducing potential from NADPH, synthesised in the pentose phosphate cycle of carbohydrate metabolism. Enzymes involved in vitamin E recycling are as follows: 1. Se-dependent thioredoxin reductase; 2.Glutathione reductase; 3.Glucose-6-phosphate dehydrogenase. Due to incomplete regeneration (the efficiency of recycling is usually less than 100%) in biological systems, the anti-oxidants have to be obtained from the diet (vitamin E and carotenoids) or synthesised in the tissues (ascorbic acid and glutathione).

It has been suggested that anti-oxidant/pro-oxidant balance in the digestive tract, in individual cells and in the whole body is responsible for regulation of many different physiological processes and ultimately responsible for maintenance of good health (Surai, 2002;Surai et al., 2003;Surai et al., 2004; Figure 3).

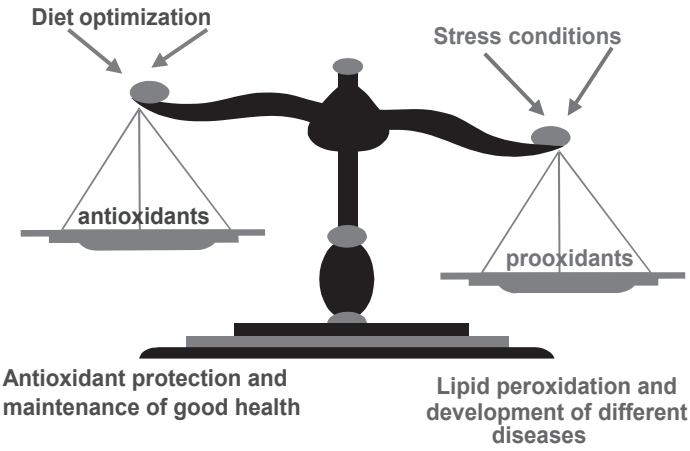

Figure 3. Antioxidant-prooxidant balance in the cell (Adapted from Surai, 2002)

Mineral anti-oxidants and their correct application, in terms of forms used and delivery systems, exert a major influence on poultry production, in particular their role in reproduction and immunocompetence.

Male fertility

The principle anti-oxidant minerals associated with male fertility are selenium and zinc. In order to be fertile, spermatozoa is characterised by high motility and acrosome integrity. To be motile, the spermatozoa needs intact mitochondria (energy-producing stations in the cell) and high membrane flexibility and fluidity maintaining membrane properties requires a high level of polyunsaturated fatty acids (Surai et al., 2003). Spermatozoa from all animal species is characterised by extremely high proportions of such fatty acids (Figure 4) and, as a result, they become very vulnerable to oxidative stress due to overproduction of free radicals. To deal with such problems the anti-oxidant system of the spermatozoa includes fat-soluble and water-soluble chain-breaking anti-oxidants as well as enzymes.

Understanding the involvement of selenium in maintenance of semen quality has been generated from data on selenoproteins. In particular, there are several selenoproteins, which are found in spermatozoa, including the enzyme GSH-Px, which are responsible for preventing damaging effects of free radicals and toxic metabolites on spermatozoa. Specific sperm nuclear GSH-Px was identified in 2001 (Pfeifer et al., 2001). It seems likely that thioredoxin reductase (TR) is also involved in anti-oxidant defence in spermatozoa, but this has yet to be confirmed. A specific sperm capsular

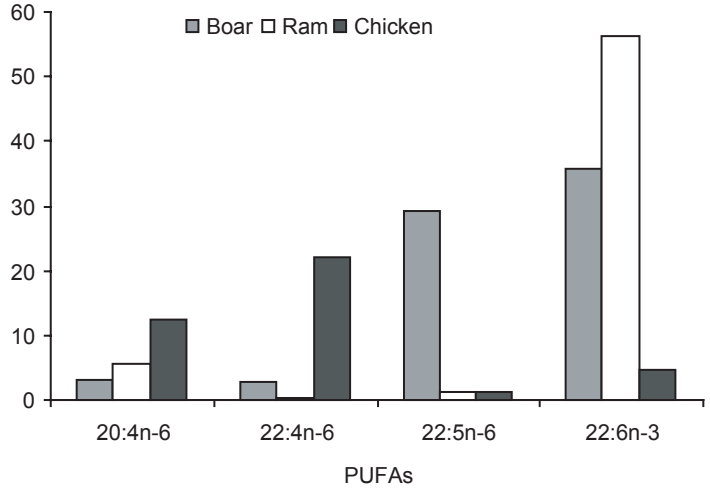

Figure 4. Polyunsaturated fatty acids (PUFAs) in semen (Adapted from Surai, 2003)

selenoprotein, located in the midpiece of spermatozoa, has recently been identified as a form of Se-dependent GSH-Px. Mitochondria, a main source of free radicals due to energy production in the spermatozoa, are located in the midpiece, making anti-oxidant protection in this location of the cell a crucial factor for motility and effective fertility. This has been demonstrated in studies where Se-deficiency has been shown to cause sperm abnormalities in this region (Surai, 2002), resulting in decreased fertilising ability. Organic selenium dietary supplementation was seen to be more efficient in improving semen morphology than selenite in these cases (Edens, 2002). Experiments with Sel-Plex in cockerel diets has further proved that organic selenium increases duration of fertility (Agate et al., 2000).

Zinc is an important mineral for male fertility maintenance. As mentioned above, zinc is an integral part of superoxide dismutase, an enzyme of the first level of anti-oxidant defences. Zinc deficiency is associated with decreased testosterone levels, sperm count, motility and production and supplemental Zn is proven to be helpful in treating male infertility (Sinclair, 2000). The role of zinc in mammalian spermatozoa can be presented as follows (Hidiroglou and Knipfel, 1984):

- Semen and its constituents contain comparatively high zinc concentration
- Zinc deficiency results in retarded development of testicular growth with marked atrophy of tubular epithelium and reduced zinc contents of testis, epididymis, and dorsolateral prostate.
- Zinc deficiency decreases output of pituitary gonadotrophins and androgen production, and zinc turnover involves testosterone as well as pituitary hormones.

- Metabolic regulation of sperm is mediated through zinc as a regulator of enzyme activity in the semen.
- Within spermatozoa, zinc is closely associated with sulfhydryl groups and disulfide linkages and is concentrated in the tail.
- Zinc in seminal plasma influences the oxygen consumption of the spermatozoa and, nuclear protein activity (Wong et al., 2000).
- Control of motility of sperm by zinc involves control of energy utilization through ATP systems involved in contraction and through regulation of phospholipid energy reserves.

Indeed, reproductive failures in the female and in spermatogenesis are manifestations of zinc deficiency (Hidiroglou et al., 1979). Involvement of dietary minerals in male reproduction is shown in Figure 5.

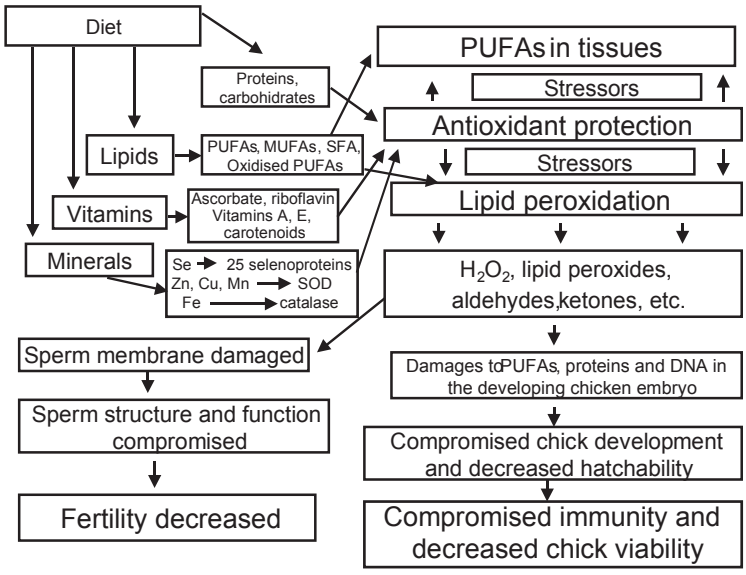

Figure 5. Nutrition, antioxidant defences and poultry reproduction

Embryonic development

Chick embryo tissues contain a high proportion of polyunsaturated fatty acids in the lipid fraction. Tissues of newly hatched chicks express a range of lipid defences, including natural anti-oxidants and enzymes (superoxide dismutase, glutathione peroxidase and catalase) as well as mineral cofactors (Se, Zn, Mn and Fe) (Surai, 2002). Se, Cu, Fe and manganese are delivered from the maternal diet via the egg and the others are synthesised in the tissues.

It is necessary to underline that maternal diet composition is a major determinant of anti-oxidant system development during embryogenesis and in early postnatal development. Minerals such as selenium are transferred from feed into egg yolk and further to embryonic tissues. Research indicates that increased supplementation of the maternal diet can substantially increase Se concentrations in developing chick tissues and significantly decreases susceptibility to lipid peroxidation. A positive effect of Se supplementation of the maternal diet has been observed at day 5 and 10 post-hatch when vitamin E concentrations in the liver and plasma were significantly elevated compared to controls. Recent results indicate that increased concentration of Se in the egg was associated with increased Se concentration in the chicken liver until 3 weeks posthatch and in the breast muscle and plasma Se concentration was elevated during 4 weeks of the postnatal development of the chicken.

Postnatal development of the chick is associated with changes in the anti-oxidant defence strategy. The main protection from oxidation in newly hatched chicks is afforded through high concentrations of natural anti-oxidants, mainly vitamin E and, in wild birds, carotenoids in tissues. However, during the first 10 days post-hatch, vitamin E and carotenoid concentrations in the chicken liver decreases 20-fold; and the same is true for turkeys, ducks and geese. To compensate for this decrease, activity of GSH-Px in the liver significantly increases. As a result, this Se-dependent enzyme becomes the major anti-oxidant defence during postnatal development of the chicken. There is some evidence to show that under commercial conditions, inclusion of organic selenium into the breeder's diet is associated with improved hatchability. Furthermore organic selenium supplementation of the maternal diet decreases chick mortality for the first two weeks posthatch confirming the relationship between anti-oxidant defences and chicken viability (Surai, 2002).

Immune system

The immune system of the animal is based on natural 'innate' (inherited from the mother) and adaptive or 'acquired' (formed through experience of pathogen exposure) immunity. Innate immunity is dependent on the efficient function of phagocytic cells, namely neutrophils and macrophages (Figure 6). These cells are equipped with an array of microbicidal weapons, such as proteases, that hydrolyse protein, disrupting membranes, and are stored in granules in the cell cytoplasm. Furthermore, these cells have a powerful system for generating large amounts of ROS and they use them as an effective weapon to destroy pathogens. However, on

release from the storage, phagosome organelle, the same free radicals can damage biological molecules, compromising phagocyte function and reducing acquired immunity. Phagocytes also produce communication molecules (e.g. eicosanoids, cytokines) that are used for signalling between various immune cells, so protecting their function is essential to other areas of immune function as well.

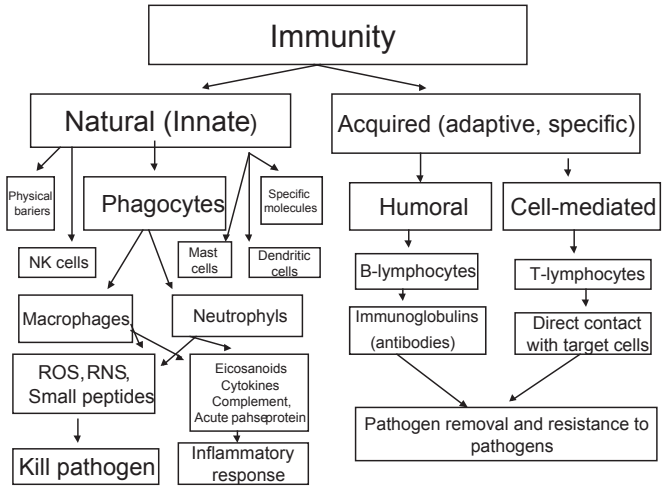

Figure 6. General scheme of immune system (Adapted from Surai, 2002)

Invading pathogens are controlled by both the innate and acquired branches of the immune system. The establishment of acquired immunity is not sufficiently fast to eradicate micro-organisms encountered by the young animal, and innate immunity is mainly responsible for recognition of invading pathogens by specific receptors. Binding of pathogens to recognition receptors induces the production of ROS and RNS, pro-inflammatory cytokines and communicating molecules which are responsible for sending regulatory signals to the acquired immune system (Werling and Jungi, 2003).

Acquired immunity is based on the activity of B- and T-lymphocytes, which produce antibodies to specific invading substances (B-lymphocytes) or bind (T-lymphocyte) and remove them from the cell. Specific acquired immune responses rely on the major histocompatibility complex's (MHC) recognition of peptide antigens taken from the pathogen to activate a variety of T cells (helper and cytotoxic cells) that interact with B cells to produce antibodies (Castle, 2000).

Acquired immunity is characterised by the ability to recognise up to 10^{11} distinct structures and is tightly regulated to turn on or off a

response. The aim is to eradicate pathogens without destroying the animal's own cells. In the healthy animal resistance to infection relies on a balance between the innate and acquired immunity. Regulation of the immune system is extremely complex, and it is only recently that science has started to understand the co-ordination of the body's response to disease. It seems likely that communication between immune cells is a crucial factor of immunocompetence.

If we imagine that immune system is a fighting force against pathogens than we would expect them to have suitable communications systems to receive and send signals to each other to ensure a correct response. Key immune cells, such as macrophages, neutrophils, T- and B-lymphocytes, have receptors on their surface that perform this function. These receptors are extremely sensitive to communicating molecules, but they are also sensitive to free radicals and can be easily damaged, potentially limiting or damaging the communication system. In such a situation without proper communication the immune system becomes useless. Indeed, immune cells may begin fighting each other, eventually destroying immunocompetence and causing autoimmune reactions.

Immune cells require chemicals to destroy the pathogens they are fighting as well as protection to ensure they do not destroy themselves. Immune cells protect themselves with natural anti-oxidants such as Se-GSH-Px and thioredoxin reductase. Macrophages destroy pathogens using an overproduction of free radicals, which can cause damage to specific enzymatic systems resulting in decreasing efficiency of the immune cell with each oxidative burst and result in apoptosis.

Se- and vitamin E deficiencies are associated with reduced functions of both innate and acquired immunity. In particular phagocytic functions, lymphocyte proliferation and antibody production may be compromised (Surai, 2002). Se supplementation has been shown to improve immunocompetence and increase resistance to various diseases. This has been demonstrated in a variety of animals including poultry, cows, sheep, horses, pigs, fish, cats and dogs. A summary of the effect of a compromised anti-oxidant system on the immune system is shown in Figure 6.

The importance of a delicate balance of the immune system is reflected by observations showing that an over-reacting immune system has similar detrimental consequences to immuno-suppression. For example, in some individuals, the immune system recognises host antigens as "non-self", attacking them and producing tissue damage leading to chronic inflammatory or autoimmune

diseases. The immune system can also become sensitised to usually benign antigens from the environment causing allergies (Calder, 2001). It seems likely that in these immune system impairments miscommunication between immune cells plays a crucial role, and protection afforded by mineral-dependant systems in essential.

The immune system is functionally immature at birth. Therefore postnatal development of the immune system is associated with accumulation of polyunsaturated fatty acids and a need for anti-oxidant protection. Therefore, expression of selenoproteins in immune cells in early development is a crucial factor in a regulation of the immunocompetence development. However, selenium reserves in the newly born or hatched animals are very limited when inorganic selenium is used in the maternal diet. In contrast, organic selenium, for example in the form of Sel-Plex, is shown in a number of studies to be able to significantly increase Se concentration in colostrum, milk and egg. The supply of selenium is absolutely essential for the formation of effective anti-oxidant defences, resulting in effective immune system maturation and immunocompetence.

It is proven that such anti-oxidant compounds as vitamin E, selenium and carotenoids are involved in immunomodulation (Surai, 2002). However, the levels of the dietary anti-oxidants showing those effects are usually several times higher than that necessary for chicken growth and development. Furthermore, other trace minerals, including Zn, Cu and Fe are also involved in immune system regulation as enzyme components.

Again, zinc is required as a catalytic, structural and regulatory ion for enzymes, proteins and transcription factors, and is thus a key trace element in many homeostatic mechanisms of the body, including immune responses. Low zinc ion bioavailability results in limited immuno-resistance to infection in aging (Ferencik and Ebringer, 2003). It is necessary to underline that a variety of in vivo and in vitro effects of zinc on immune cells depend on its concentration, e.g. major immune cells show decreased function after zinc depletion. In monocytes especially, all functions are impaired, whereas in natural killer cells, cytotoxicity is decreased, and in neutrophil granulocytes, phagocytosis is reduced (Ibs and Rink, 2003). Furthermore, the normal functions of T cells are impaired and B cells undergo apoptosis. Impaired immune functions due to zinc deficiency are reversed on supplementation. However, high dosages of zinc have negative effects on immune cells and show alterations that are similar to those observed with zinc deficiency (Ibs and Rink, 2003). Organic Zn is characterised by

improved availability in comparison to inorganic sources and is considered to be beneficial for animal health.
The immune system requires copper to perform several functions, but little is known about its direct mechanism of action (Percival, 1998). For example, some of the recent data from various studies showed that interleukin 2 is reduced in copper deficiency and is likely the mechanism by which T cell proliferation is reduced. It is important to note that even in marginal deficiency, when common indexes of copper are not affected by the diet, the proliferative response and interleukin concentrations are still reduced (Percival, 1998). Copper deficiency is also associated with a decreased number of neutrophils. Their ability to generate superoxide anion and kill ingested micro-organisms is also reduced in both overt and marginal copper deficiency. In many experiments it has been proven that Cu deficiency reduces antibody production, although cell-mediated immunity is more resistant to Cu deficiency. However, Cu deficiency appears to reduce production of interferon and tumour necrosis factor by mononuclear cells (Spears, 2000). Inorganic copper has a strong pro-oxidant effect and if not bound to proteins could stimulate lipid peroxidation in feed or even more importantly in the intestinal tract. Organic copper does not possess pro-oxidant properties and can improve the copper status of animals

Iron is a vital metal for the proliferation of all cells including those of the immune system. Indeed iron plays an essential role in immuno-surveillance, because of its growth promoting and differentiation-inducing properties for immune cells as well as its interference with cell mediated immune effector pathways and cytokine activities (Weiss, 2002). Iron is also crucial in the proliferation of tumour cells and micro-organisms, as it is involved in mitochondrial respiration and DNA synthesis. Iron deficiency causes several defects in both the humoral and cellular immunity. One of the most profound changes is a reduction in peripheral T cells and atrophy of the thymus (Bowlus, 2003). Growing evidence suggests that T cells may regulate iron metabolism perhaps through interactions with the non-classical major histocompatibility complex gene HFE.

Iron is a very strong pro-oxidant and, if not bound to proteins, can stimulate lipid peroxidation. This is especially relevant to digestive tract where lipid peroxidation can cause enterocyte damage and decreased absorption of nutrients, particularly those with anti-oxidant properties. If iron is included in a premix in inorganic form it can stimulate vitamin oxidation during storage. Therefore organic iron supplementation is a solution to avoid such problems and improve iron status of animal.

Disease resistance and improvements in immune response via anti-oxidants

The main goal in improving the immune system is to increase resistance to various diseases, and this area has been extensively studied with chickens. Feeding a combination of vitamin E with Se has been known to reduce mortality and increase body weight gain in chickens infected with *Eimeria tenella* (Colnago *et al*., 1984). The same authors showed that dietary supplementation with selenium or vitamin E reduced mortality and increased body weight gain of non-immunized chickens infected with *E. tenella* in three out of four experiments. When chicks were inoculated with virulent Marek's disease virus at 10 days of age, selenium (dosed at 0.6 mg/kg) decreased the morbidity and mortality of the flock. In particular, selenium has been demonstrated to increase the ability of cells to remove ROS and lipid peroxides, and decrease the degree of tissue damage caused by ROS (Huang and Chen, 1996). Another experiment examined the effect of anti-oxidant supplementation against Infectious Bursal Disease (IBD). One day old chicks were fed on a diet containing selenium at 0.086 mg/kg, 0.3 mg/kg or 0.6 mg/kg. The chickens were infected with IBD virus at 39 days of age. Ten days later the mortality rates for the 0.086, 0.3 and 0.6 mg/kg diets were 33.3% 12.4% and 10.6%, respectively. Infection-induced inhibition of T lymphocyte transformation was lower in the selenium supplemented birds (Bu *et al*., 1996).

In other trials, when Se was added to the feed of White Leghorn chickens prior to challenge with either *E. coli* or sheep erythrocyte antigen, the incidence of death or lesions was reduced from 86% to 21% at the optimal dose of Se (0.4 mg/kg feed; Larsen *et al*., 1997). Lower Se values have been measured in birds infected with *Ascaridia galli* compared with controls, and this has been related to a lower degree of Se absorption, and the regeneration of the intestinal mucosa in infected birds (Damyanova *et al*., 1995).

Nutritional strategies to prevent oxidation damage

Animals are capable of synthesising anti-oxidant enzymes, but dietary availability of Se, Mn, Zn and Cu are among major restrictions for this synthesis. For example, recently it has been shown that availability and efficiency of selenium in animal nutrition greatly depends on the dietary sources of this element. Indeed, animal dietary formulation is based on the supplementation with selenium as a safety margin to prevent selenium deficiency and to maintain good health and high reproductive performances of animals. Recently it has been suggested that, during evolution, all

animals have adapted only to use the organic form of selenium (Surai, 2002). Indeed all feed ingredients contain selenium only in organic form, mainly as various seleno-amino acids. This means that inorganic selenium (selenite or selenate) is not a suitable substitute. There is a principal difference in absorption and metabolism between these two forms of selenium. Organic selenium is actively absorbed in the intestine as amino acid employing similar selective uptake mechanisms as other amino acids. In contrast, inorganic selenium is passively absorbed as a mineral.Because of these differences in uptake only selenomethionine can build Se reserves in the body (mainly in muscles). Since selenomethionine is not synthesised in animals, only plants, there are no Se tissue reserves formed when inorganic selenium is used. Other forms of seleno-aminoacids (for example, Se-cysteine) are not a reserve form of the element. This principal difference can explain why organic selenium is more effective than inorganic one, especially in stress conditions, when uptake efficiency is crucial.

To compare and contrast commercial situations, we can consider two different scenarios of anti-oxidant defence in poultry. The first, and most common, scenario is for animals fed inorganic selenium in the diet. Under physiological stress, the body responds by using anti-oxidant reserves in the body and, more importantly, by synthesising additional selenoproteins (Figure 7). In this case the main limitation is the absence of tissue selenium reserves and a restricted ability to synthesise additional selenoproteins, resulting in inadequate anti-oxidant protection when overproduction of free radicals is encountered. In this scenario we would expect the immune system, general health and reproduction to be compromised.

It is necessary to realise that dramatic differences in physiological stress are not required to cause such events. Sometimes a small difference, which is difficult to notice, can trigger enough radicals to cause a major problem. Indeed several cumulating, consecutive stresses may dramatically affect animal behaviour and health. This is especially important for newly born animals including birds, since their anti-oxidant mechanisms are not mature and they are dependent on anti-oxidant maternal delivered via the egg (birds) or colostrum and milk (mammals). Since inorganic selenium is not well transferred to the egg and even less effective in transferring to the milk we could not expect an anti-oxidant system improvement through this route in this scenario.

The alternative scenario would be when organic selenium is fed (Figure 8). This leads to selenium reserves being accumulated in

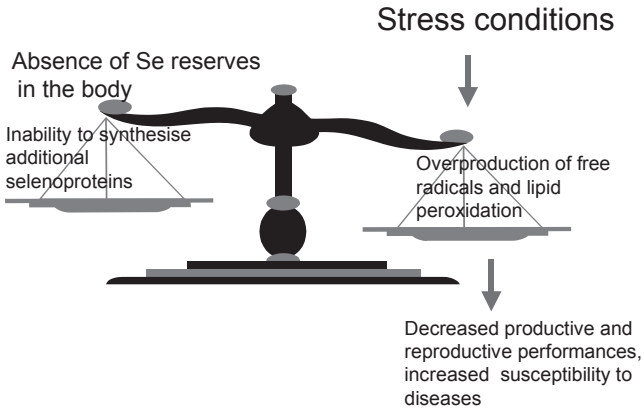

Figure 7. Inorganic selenium scenario in poultry production (Adapted from Surai, 2002)

the form of selenomethionine in tissue. Under stress conditions, protein catabolism by proteosomes releases Se, which can be used for the synthesis of additional selenoproteins to prevent any damaging effects of free radical overproduction. This is especially important since many stresses are associated with a decrease in feed consumption. As a result of such syntheses, selenoproteins can reduce lipid peroxidation, benefiting animals by maintaining immunocompetence and reproductive performance. As selenomethionine is transferred to the egg and to the milk and colostrum, newly hatched chicks or new-born animals benefit as a result of their improved anti-oxidant system.

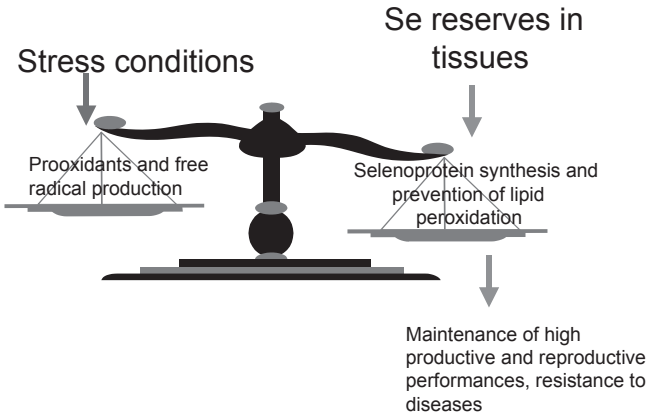

Figure 8. Organic selenium scenario in poultry production (Adapted from Surai, 2002)

It is important to realise that this scenario has limitations in terms of level of stresses we are considering. For example, when high levels of toxins are present in the diet or environmental stresses are high, the body response may not be sufficient to prevent pathobiological changes in animal body. On the other hand, this model can be effective for a range of everyday stress conditions.

The development and commercial application of products such as Sel-Plex, which have guaranteed composition and proven efficacy shown in research and commercial trials opens a new era in animal nutrition. It provides opportunities not only for improvement of animal health and productivity but also for production of Se-enriched meat, milk, eggs and other foods that can improve human diets. A comparison between Sel-Plex and selenite, based on published data, clearly showed advantages of the natural form of Se in comparison to selenite (Table 2). Therefore, it seems appropriate that selenite be considered as a drug, and should be used accordingly. For example, when Se deficiency is diagnosed based on clinical signs, selenite would be the preparation of the choice. Using it via feed, water or injection will solve the short-term or acute Se deficiency, which has been demonstrated under various experimental conditions with chickens, pigs and cattle. The beneficial effect of organic selenium for animals can be even higher when it is used in combination with other organic minerals, namely Zn, Cu, Fe and Mn. It has been proven that these minerals are more effectively absorbed and metabolised in the body and this could be a major advantage for poultry, pig, dairy and beef industries. General relationships between anti-oxidants with fertility and development in poultry are shown in Figure 5.

Conclusions

Research has shown that we are living in the world of free radicals. Humans, chickens, pigs, cows and all other animal species are exposed to free radical attack in everyday life and that is why an integrated anti-oxidant system has evolved in every cell to prevent damage to biologically important molecules including DNA, proteins and lipids. Some anti-oxidants are synthesised in the body, however diet forms the major source of anti-oxidants. From the hundreds of dietary compounds possessing anti-oxidant activities, selenium, zinc, iron, manganese and vitamin E are considered to be the core of anti-oxidant defence. It has been appreciated that the efficiency of anti-oxidants depends on their form in the diet, and in recent years it has been proven that organic selenium (e.g. Sel-Plex) has important advantages in comparison to inorganic selenium.

Benefits of organic selenium have been proven for many species, including chicken, pigs, cows, sheeps and fish. It seems likely that an optimal combination of organic selenium and vitamin E in the diet is a key for an effective anti-oxidant defence. However, there is a need for further research in this field to establish those optimal combinations for each species depending on age, productivity and other relevant technological conditions. Inorganic copper and iron

Table 2. Major differences between organic selenium (Sel-Plex™) and selenite

Parameter	*Organic selenium (Sel-Plex™)*	*Selenite*
Absorption	Similar to Methionine with active transport in the gut	Similar to other mineral with passive transport in the gut
Accumulation	Building Se reserves by non-specific incorporation of SeMet into the proteins	Not accumulated in the body
Toxicity	At least 3 times less toxic than selenite	Highly toxic, can penetrate via skin causing problems
Bioavailability	Higher bioavailability in comparison to selenite to animals and human	Very low availability for ruminants due to reduction by rumen microbes
Anti-oxidant activity	SeMet possess anti-oxidant properties per se and could scavenge NO and other radicals	Possess pro-oxidant properties and could stimulate free radical production when reacting with GSH
Effect on DNA	SeMet stimulate DNA-repair enzymes	Selenite can cause DNA damage
Transfer to eggs, milk and meat	Transferred to egg, milk and meat giving a possibility to produce designer/ functional food	Poorly transferred to eggs, milk and meat
Transfer via placenta	Better transferred via placenta than selenite	Poorly transferred via placenta
Reactions with other elements	Neutral, ascorbic acid promotes SeMet assimilation from the diet	Highly reactive, reduced to metallic, unavailable selenium by ascorbic acid
Protective effect in stress conditions	Provide additional protection due to Se reserves in the body	Cannot provide additional protection due to absence Se reserves in the body
Effect on drip loss	Did not affect drip loss	Increases drip loss
Environmental issues	Better retention in tissues, less released with faeces and urine	Low retention in tissues and high release with faeces and urine
Stability during storage and feed processing	Stable	Stable
Classification based on the mode of action	Feed additive	Drug

are major stimulators of lipid peroxidation in digestive tract, using organic forms of these elements can avoid their detrimental activity and help maintaining high productive and reproductive performance of poultry and farm animals reared under commercial conditions.

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