THE EFFECTS OF DIETARY SELENIUM INTAKE AND LIPOPOLYSACCHARIDE STIMULATION ON SELECTED IMMUNE AND INFLAMMATORY MARKERS IN C57BL/6 MICE

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ABREVATIONS

AAS	Atomic absorption spectrophotometry
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Aminotransaminase
CRP	C - reactive protein
Cys	Cysteine
DCs	Dendritic cells
DI-1	Deiodinase-1
DI-2	Deiodinase-2
DI-3	Deiodinase-3
FACS	Fluorescence activated cell sorter
FNB	Food and Nutrition Board
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
ICP-MS	Inductively coupled plasma mass spectrophotometry
IFNγ	Interferon gamma
Ig	Immunoglobulin
IKβα	Inhibitory kappa beta alpha
IL-12	Interleukin-12

INAA	Instrumental neutron activation analysis
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
Met	Methionine
NBF	Neutral buffered formalin
NF-κβ	Nuclear factor kappa beta
NHANES	National health and nutrition examination survey
NKC	Natural killer cells
NOAEL	No observed adverse effect level
NO	Nitric oxide
ONOO	Peroxynitrite
PBS	Phosphate buffered saline
PLGSH-Px	Phospholipid glutathione peroxidase
RDA	Recommended dietary allowance
REDOX	Reduction oxidation
ROS	Reactive oxygen species
Se	Selenium
SeCys	Selenocysteine
SeMet	Selenomethionine
SPS2	Selenophosphate synthetase 2
T_4	Thyroxine
T_3	Triiodothyronine
TG	Triglycerides
Th	T-helper

TMB	3,3’,5,5’,- Tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TPN	Total parenteral nutrition
TR	Thioredoxin reductase
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Selenium an Essential Trace Element

The understanding that selenium (Se) is an essential trace element has come from research over the past several decades. A historical year was marked in 1957, for Se to be recognized as an essential trace element as a result of being a component of Factor 3 which prevented liver necrosis in rats (Schwarz and Foltz 1957). Later in 1973, a graduate student from University of Wisconsin discovered that Se is part of glutathione peroxidase (GSH-Px) (Rotruck, Pope et al. 1973) and in 1985, phospholipid hydroperoxide glutathione peroxidase (PLGSH-Px) was identified as a second Se containing enzyme (Ursini, Maiorino et al. 1985). These two enzymes generally characterize the biochemical functions of Se such that the presence or absence of Se affects the underlying mechanism by which Se contributes to redox balance. In animals, Se deficiency resulted into pathological conditions manifested as defective growth, hepatic necrosis, myocardial degeneration and muscular dystrophy (Hoekstra 1975).

Deficiency of Se induces, as a result of a high level of free radicals, reactions contributing to pathological conditions such as diabetes, cardiovascular disease, hypertension and related complications (Sunde 2006). Se is an essential component of the

antioxidant enzyme GSH-Px, which functions to catalyze a reaction that scavenges reactive hydrogen peroxide and lipid hydro peroxide and converts them to less reactive end products (Puri 2002).

However, before Se was identified as an essential element, it was well known for its chronic toxic effects throughout history. Short term exposure to excess Se in animals resulted in abnormal posture, unsteady gait and eventual death while chronic exposure resulted in "blind staggers", and "alkali disease" of livestock as a result of eating highly seleniferous plants (James and Shupe 1984). According to these authors, "blind staggers" is characterized by blindness, weakened legs and paralysis while "alkali disease' is manifested by dullness, anorexia, weight loss, ataxia, and dystrophic hooves" (James and Shupe 1984). Humans living in Se excess areas also developed changes in their integumentary system manifested as dermatitis, hair loss and nail changes (Yang, Wang et al. 1983). These changes were observed at an intake greater than 16 times the optimal level for the recommended dietary allowance (RDA) (Yang, Wang et al. 1983). As a result, early research on Se focused on toxic manifestations of excess Se intake. Current research, however, also includes focus on Se deficiency and the resulting pathophysiological conditions after Se was identified as an essential nutrient for balancing the reduction and oxidation (redox) system in the host (Rahul and Geeta 2007). The emerging evidence is promising in supporting the importance of Se in chronic diseases and its impact on the immune system (Rahul and Geeta 2007).

Selenium in the Immune System

Overview of the Immune System

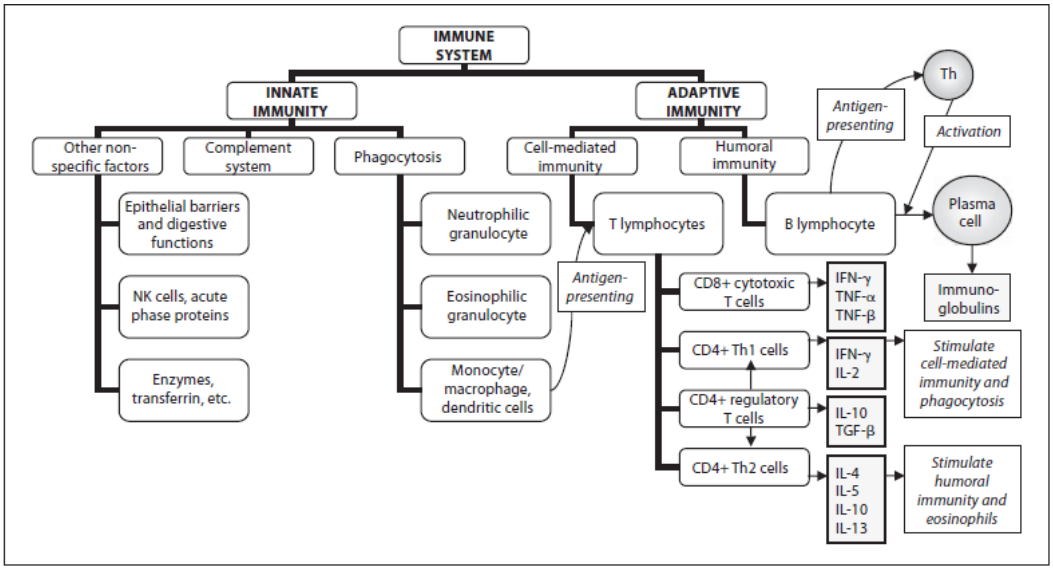
The body fights disease organisms, cancers, and foreign substances by its immune system. The immune system is generally divided into two interactive parts named as innate or non-specific immunity and adaptive or specific/acquired immunity (Parkin and Cohen 2001).

Innate cellular immunity includes cellular elements consisting of macrophages, leukocytes, natural killer cells (NKC) and dendritic cells (Delves and Roitt 2000) (Fig.1). The innate immunity also includes some components with recognition molecules such as C - reactive protein, serum amyloid protein and mannose-binding protein as acute phase proteins and helps activates the complement system for phagocytosis and cell lysis (Delves and Roitt 2000). These molecules help to distinguish host cells from invaders and facilitate phagocytosis and removal of the intruder. Secretions of pro-inflammatory cytokines (IL-1, IL-6, IL-12; TNFα,) leukotrienes, prostaglandins and reactive oxidative species (ROS) are increased by stimulated phagocytic cells (Ryan-Harshman and Aldoori 2005). The NKC lyse cancerous cells and pathogen-infected cells in response to macrophage-driven cytokines and interferons which help to arrest infections (Delves and Roitt 2000). The dendretic cells (DCs) are also activated by interferon-γ (IFNγ) and serve as antigen-presenting cells and activate naive T cells to initiate immune responses in the absence of formulated immunological memories of the antigen (Ryan-Harshman and Aldoori 2005).

Adaptive immunity is a defense system that strengthens innate immunity (Fig.1.1) (Parkin and Cohen 2001). When infection occurs for the second time, the B and T memory cells quickly activate the immune system (Parkin and Cohen 2001). The T lymphocytes represent the major portion of the cells of specific immunity (Delves and

Roitt 2000). T lymphocytes originate in bone marrow and mature in thymus while B lymphocytes originate and mature in bone marrow (Delves and Roitt 2000). Both types of lymphocytes have receptors that differentiate self from non self and identify antigens specific to infective agents (Delves and Roitt 2000). On the other hand, humoral immunity is facilitated by antibodies secreted in B- cells and this immunity is highly protective against extracellular pathogens (Albers, Antoine et al. 2005) . The antibodies bind with antigen on the surface of pathogens and facilitate destruction by macrophages (Albers, Antoine et al. 2005).

Fig.1.1: Overview of the Immune System (Wintergerst, Maggini et al. 2007)



The role of Selenium in immune function

The immune system is dependent upon several processes which include production of reactive oxidative molecules (i.e. protection against microbial pathogens), organized and coordinated functions of adhesion molecules and production of soluble mediators such as eicosanoids and cytokines and receptors (McKenzie, S. Rafferty et al. 1998). Se likely influences these immune processes at all stages as it is important for

optimum function of both the innate and adaptive immune systems (McKenzie, S. Rafferty et al. 1998). The production of ROS is important for microbicidal activity of immune cells, such as neutrophils, as released in the respiratory burst reaction (McKenzie, Arthur et al. 2002; Arthur 2003). Excessive production of ROS, however, is lethal. In small amounts, the ROS help to attack microbial agents by generating inflammation, but excessive and prolonged generation of these reactive species may cause damage to the host (McKenzie, Arthur et al. 2002; Arthur 2003; McKenzie, Beckett et al. 2006). The antioxidant system of the host is used as a defense against excessive ROS.

The first evidence on the role of Se in immune function was derived, in 1959, from a study in dogs injected with ^{75}Se which incorporated the isotope into a leukocyte protein (Hoffmann 2007). The protein which was observed then was later identified to be cytoplasmic glutathione peroxidase (cGSH-Px) (Rotruck, Pope et al. 1973). In sheep and in humans, Se has been found to be concentrated in tissues such as spleen, liver and lymph nodes which are involved in immune response (Spallholz 1990).

The finding in 1973 that Se was required for the activity of the selenoenzyme cGSH-Px provided some insight into a mechanism by which Se exerted its biological functions including its impact on the immune system. cGSH-Px detoxifies harmful ROS such as organic hydroperoxides, as well as hydrogen peroxide, which are produced during cellular respiration (Spallholz 1990; Sunde 1990; Arthur, Bermano et al. 1996; Foster 1997; Rayman 1997). Other types of GSH-PXs, as well as other selenoenzymes and selenoproteins also play preventive roles against oxidative damage to cells in the body (Spallholz 1990).

Throughout the 1970's and the 1980's, there was marked progress in research on the immunostimulatory effects of Se, as summarized by Spallholz (Spallholz 1990). Research in the 1980's demonstrated the immunological protective roles of Se through modulation of antibody and complement production. Research has shown that Se intensifies immunological responses to several types of immunogens such as tetanus toxoid, typhoid toxin and sheep red blood cells (Spallholz 1990). On the other hand, when Se was deficient in the host, it has been associated with failure of neutrophil responses, reduction of neutrophil numbers, reduced antibody production to sheep red blood cells, enhanced H_2O_2 discharge by phagocytes, decreased antibody titers to bacterial and mycotic antigens and decreased natural killer cell activity (McKenzie, Beckett et al. 2006). Se supplementation as sodium selenite in drinking water (2.5µg of Se/ml), on the other hand, boosted the immunity from vaccination against malaria by increasing antibody-producing B-cell numbers and T-cell dependent antibody production with elevated concentration of Se in neutrophils and GSH-Px activity in lymphocytes (Desowitz and Barnwell 1980). In some instances, however, toxic levels of Se supplements have been shown to decrease immunity (Spallholz 1990), which probably indicates the need for an optimal dose of Se for enhanced stimulation of the immune system.

The current studies also show that adequate dietary Se is essential for both innate and adaptive immune responses (Wang, Wang et al. 2009). Se deficiency affects several immune response pathways including impairment of leukotriene B_4 synthesis, which assists in neutrophil migration to inflammatory sites (Arthur 2003). Similarly, a decrease in the humoral immune response (immunoglobulin production) was shown in Se

deficiency both in rats and humans (Arthur 2003). For example, in Se deficiency, markers of the humoral immune system such as IgM, IgG and IgA titers were decreased in rats, while IgG and IgM titers were found to be lower in humans (Arthur 2003).

Overall, the role of Se, as an essential nutrient, for immune response is well recognized both in animals and humans (McKenzie, S. Rafferty et al. 1998; Arthur 2003). The GSH-Px facilitates the antioxidant function of Se to minimize harmful effects of lipid hydroperoxides and hydrogen peroxide (Arthur 2000). Different peroxidases function in different parts of cells and tissues (Arthur 2000; Pfeifer, Conrad et al. 2001). For example, the GSH-Px functions in the extracellular space, the cell cytosol and in cell membranes as in the gastrointestinal tract and influences the immune response of the host. In addition, the thioredoxin reductase (TR) (Miller, Walker et al. 2001), and selenoprotein P and W also provide antioxidant functions (McKenzie, Arthur et al. 2002; McKenzie, Beckett et al. 2006). All selenoproteins with antioxidant functions have roles in the immune system (McKenzie, Arthur et al. 2002). As these selenoproteins are present in all cells, it may be possible that Se affects cellular activities through antioxidant functions and regulation of the redox-active proteins (McKenzie, Beckett et al. 2006). Thus, Se has a role in the control of several metabolic functions and specific processes that enhance the immune system. The specific immune challenges, however, determine which functions of Se will be involved in the immune response.

From the studies discussed above, information on the specific dose of Se recommended to promote optimal immune response is lacking. After the first study on the role of Se in the immune system in 1959, several studies were undertaken to establish the relationship between Se and immune response both in animal and human studies.

Most of the studies used different chemical forms and doses of Se which made it difficult to interpret results and draw conclusions. This study, as part of an experimental study on selenium and bone, thus, has assessed the immune response of mice stimulated by low dose and slow release lipopolysaccharide (LPS) and supplemented with dietary Se to investigate the effect of different doses of dietary Se supplementation as sodium selenate on immune response.

We used slow-release Lipopolysaccharide (E. coli Serotype 0127) pellets to provide a consistent dose of LPS for 28 days (Innovative Research of America, Sarosota, FL) and these pellets were implanted using the method of Smith et al (Smith, Lerner et al. 2006). This provided low grade inflammation and we measured selected inflammatory and immune markers in mice supplemented with dietary Se. Based upon the evidence outlined above, Se modulates inflammation in several ways. Accordingly, several studies have been carried out to assess the extent to which Se down-regulates excessive inflammatory responses to prevent further impacts of inflammation. Most of these studies used high grade inflammation models and to our knowledge, no prior study has been carried out to assess the impact of Se on low grade inflammation. Therefore, the purposes of this study were first to determine if increasing levels of Se prevented LPS-induced alterations in numbers of selected immune cells and in biochemical markers and secondly if these effects of Se were associated with alterations in expression of selected pro-inflammatory cytokines.

Overall Objective

The overall objective of this study was to assess selected immune markers in response to dietary Se supplementation and to low grade chronic inflammation stimulated by low dose slow - release LPS in an *in vivo* experiment.

Specific Objectives

1. Assess plasma IL-12, IFNγ and IgG2a as markers of changes in macrophage activation, as well as Th-1, and B-cell responses in plasma samples.
2. Measure GSH-Px activity in plasma collected from mice with or without slow release LPS and supplemented with dietary Se.
3. Determine the plasma total antioxidant capacity (TAC) in mice with or without slow release LPS and supplemented with dietary Se.
4. Measure bone marrow lymphocyte proliferation to detect change in the number of total T-cells, T-helper cells and B-cells in mice stimulated with time-release LPS and supplemented with dietary Se compared to dietary equivalent groups not implanted with LPS.

Hypothesis

There will be significant differences in plasma concentration of inflammatory biomarkers (IgG, IFNγ and IL-12) and oxidative stress markers (GSH-Px and TAC) in mice supplemented with different dietary Se concentrations and stimulated with time-release LPS.

Statement of the Problem

Se works by promoting both the innate and acquired immune systems (Brown and Arthur 2001). The selenoproteins are very important in regulating antioxidant and redox

systems thereby influencing membrane integrity and guarding against DNA damage (Arthur 2003). The antioxidant effect of Se is mediated through GSH-Px. It plays a role by removing free radicals produced during normal metabolism and oxidative stress. Se deficiency leads to decreased activity of GSH-Px and reduced ability to produce respiratory burst reactions by neutrophils and macrophages, which are important in killing microbes (Arthur 2003). The role of Se in up-regulating IL-2 receptors which are required for effective cellular and humoral immune responses also is well documented (Arthur 2003). Overall, the existing body of literature consistently indicates that Se has a role in maintaining the immune system. However, this evidence does not indicate the actual threshold of Se concentration required for optimum immune system function beyond its classical antioxidant functions. Considering this as a gap in knowledge, this study was carried out to investigate the role of dietary Se supplementation in mice stimulated by LPS and to identify the Se dose that enhances immune response. No previous study, to our knowledge, has examined the immune response of Se to low grade chronic inflammation.

Significance of the Study

The results of this study add knowledge in the field of Se and immunity. The findings support that the supplemental dietary Se enhances immune response. Accordingly, it is recommended that another animal study be carried out to refine doses. This study will also add knowledge on the host's immunological response to high Se intakes.

Organization of the Dissertation

The dissertation is organized first by giving an introduction on Se as an essential trace element in the first chapter. The second chapter presents a literature review with a detailed account of the metabolism of the Se in the physiological system and includes physiological functions, requirements, excretion, health impacts of deficiency, dietary sources, methods for assessment of selenium status and specific roles of Se in the immune system. The third chapter presents study design and methods used to assess impacts of Se supplementation on the immune system. The following chapters present the results of the study in the form of two manuscripts. These chapters address the immunostimulatory effects of dietary Se assessed by pro-inflammatory cytokines, plasma Se, total antioxidant capacity and GSH-Px as markers of inflammation and change in numbers of peripheral white blood cells and T and B - cells in bone marrow. The summary and conclusion chapter is followed by bibliography and appendices.

CHAPTER II

LITERATURE REVIEW

Metabolism of Selenium

Se, as a trace element, is classified as metalloid and stands in the same group with oxygen, sulfur and tellurium (Suzuki 2005). Se can be found in metallic form, inorganic forms, such as selenate and selenite and organic forms such as selenocysteine and selenomethionine (Suzuki 2005). Selenocysteine (SeCys) and selenomethionine (SeMet) are the most common organic forms found in plant and animal sources of foods (Fig.2.1) as selenoenzymes and other proteins (Foster 1997; Suzuki 2005). Selenite and selenate are also found in foods and water, although the drinking water contain insignificant amount of Se (Foster 1997; Suzuki 2005). The SeCys is present in the selenoproteins as an amino acid residue in both plants and animals foods, while SeMet is present as SeMet residue in general protein without being differentiated from methionine (Met) and exists freely to the Met pool (Suzuki and Ogra 2002).

SeCys doesn't exist freely because it contains free selenol (-SeH) and is therefore too highly reactive to exist in a free form. In plants, Se is accumulated in different non-reactive forms of amino acids such as SeMet, methyl selenocysteine (MeSeCys) and γ-

glutamyl-Se-methyl-selenocysteine (Suzuki and Ogra 2002). Plants accumulating Se are divided based on the type of Se accumulated (Suzuki 2005).

For example, broccoli accumulates selenate, while cucumbers and grain accumulate SeMet. Plants such as onion and garlic accumulate MeSeCys.

Selenium as a Component of Proteins

Se incorporates into proteins as SeCys or SeMet (Ogra, Ishiwata et al. 2004). The SeMet from dietary sources can be incorporated into proteins without discrimination between SeMet and Met (Ogra, Ishiwata et al. 2004). SeCys incorporated into proteins is not from dietary sources but is endogenously formed from selenide (Suzuki 2005). Proteins which contain SeCys are called selenoproteins while proteins containing SeMet are referred to as Se-containing proteins (Sunde 2000; Suzuki 2005). Generally, Se in these proteins is part of the gene product as a form of SeCys and SeMet residue which makes it different from other metals (Burk, Hill et al. 2003). For example, other trace elements such as copper and zinc attach to their proteins once the primary structures of the proteins are translated, while Se incorporates into protein as the amino acid SeCys during the translation of the primary structure (Burk, Hill et al. 2003; Suzuki 2005) .

SeCys incorporates into proteins (Burk, Hill et al. 2003; Squires and Berry 2008) and is encoded by a UGA codon in the selenoprotein mRNA (Squires and Berry 2008). Most of the selenoproteins are in the form of enzymes playing a catalytic role in oxidation reduction reactions with SeCys available at their active site (Hatfield and Gladyshev 2002). There is a structural similarity between SeCys and cysteine (Cys) except that SeCys contains Se in place of sulfur in cysteine (Copeland 2003).

Absorption and Transport of Selenium

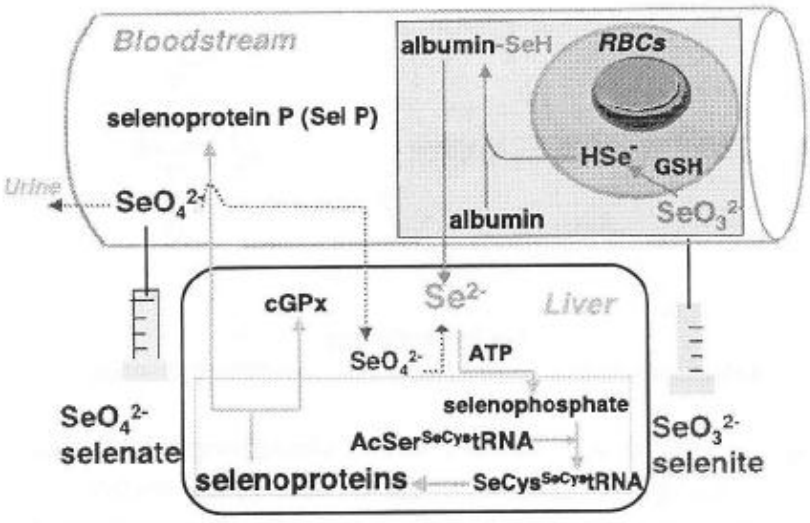
Several chemical forms of Se present in foods are well absorbed (Swanson, Patterson et al. 1991). The usual absorption rate ranges from 50 to 100%. SeMet, the major dietary form, is more than 90% absorbed by a mechanism similar to that used by Met (Swanson, Patterson et al. 1991). Not much is known about the absorption of selenocysteine but it is assumed to be better absorbed than inorganic Se (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000). Selenate absorption uses a mechanism common to sulphate, depending on the Na+ gradient, and absorption is maintained by the Na+/K+ ATPase (Navarro-Alarcon and Cabrera-Vique 2008). Selenate is absorbed almost completely but some amount gets lost to urine before it incorporates into the tissues. On the other hand, selenite absorption is said to be less consistent, due to interactions in the gut, but is well retained in the system once absorbed compared to selenite (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000). Transporters for Se across membranes are unknown. SeMet uses the same transportation mechanism used by Met.

Metabolic Pathways for Conversion of Se to a Common Intermediate: Selenide

Diet contains different chemical forms of Se and all converge into a common active intermediate for the synthesis of SeCys to be part of SeCystRNA and then to SeCys UGA codon for synthesis of selenoprotein (Glass, Singh et al. 1993). Inorganic forms of Se (selenite and selenate) are reduced by glutathione (GSH) and thioredoxin reductase (TR) to selenide (Hatfield 2001; Suzuki 2005). Selenate and selenite are transported using bicarbonate and phosphate buffer systems. Selenite is directly taken up by red blood cells (Suzuki, Shiobara et al. 1998) , while selenate ions are taken up by

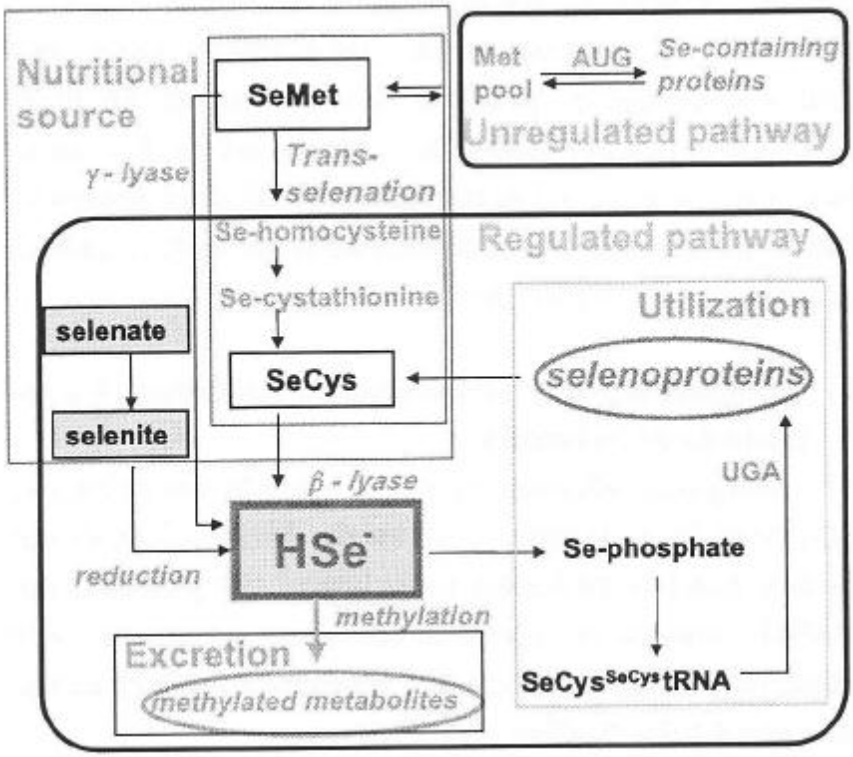
hepatocytes (Kobayashi, Ogra et al. 2001). Selenite is readily reduced to selenide, in red blood and intestine cells, and released into the blood stream (bound to albumin) to be transported to liver (Shiobara and Suzuki 1998). The reduced form of these inorganic forms of Se then is utilized by liver to synthesize selenoprotein P, which is Se transport protein and GSH-Px to be released back into the blood stream (Suzuki, Ishiwata et al. 1999).

Fig.2.1: Differences in Metabolic Pathways for Selenite and Selenate (Suzuki 2005)



On the other hand, organic forms of Se (SeCys and SeMet), as selenoaminoacids, are transformed to selenide by the lyase reaction (Schomburg, Schweizer et al. 2004). B-lysate transforms SeCys directly to selenide while SeMet transforms to selenide by a trans-selenation pathway in the cells (Fig.2.1). When there is excessive Se intake, the C-Se bond is cleaved at the γ position by γ-lyase of SeMet which results in selenide for synthesis of selenoproteins (Okuno, Kubota et al. 2001).

Fig.2.2: Metabolic Pathways for Selenium (Suzuki 2005)



Synthesis of SeCys occurs during protein synthesis and converts to selenide for the formation of selenophosphate, which is used for the synthesis of selenoproteins (Low, Harney et al. 1995; Zeng 2009). Selenophosphate synthetase is utilized to catalyze this reaction using ATP (Sunde and Evenson 1987). As dietary SeCys or SeMet couldn't directly be used for selenoproteins formation, the amino acid serine serves as a donor of carbon skeleton for SeCys (Sunde and Evenson 2002). Serine gets esterified to the 3' terminal adenosine of tRNAsec UCA to produce Ser-tRNAsec UCA by seryl-tRNA synthases (Hatfield, Choi et al. 1994). As a second step, selenocysteine synthase catalyzes a reaction that helps to substitute the serine-OH with -SeH from selenophosphate to produce selenocysteine-tRNASec UCA resulting in synthesis of SeCys (Tormay, Wilting et al. 1998). Degradation of SeCys is catalyzed by

selenocysteine lyase which releases elemental Se that converts to selenide to complete the cycle (Mihara, Kurihara et al. 2000).

SeMet doesn't have a specific codon but uses the same AUG codon used by Met (Fig: 2.2) (Butler, Beilstein et al. 1989) to be incorporated into general proteins until degraded and released to be converted by trans-selenation or direct γ-lyase pathways to selenide (Fig.2.1) The concentration of SeMet in the general body protein is proportional to the concentration of Se in the food.

Mammalian Selenoproteins

In 1973 the first SeCys containing protein in mammals was recognized (Rotruck, Pope et al. 1973). Since 1973, more types of selenoglutathione peroxidases (GSH-Px) have been identified (Hatfield and Gladyshev 2002) and characterized. Of these, the GSH-Pxs protect cells against oxidative damage by reducing hydrogen peroxide and hydro-peroxides (Flohé and Brigelius-Flohé 2006). The phospholipid glutathione peroxidases (PLGSH-Px), also function in the reduction of phospholipid (Flohé and Brigelius-Flohé 2006), cholesterol, and cholesteryl ester hydroperoxides to prevent cell membrane lipid peroxidations. The PLGSH-Px also has another structural function in male spermatozoa and this may be the reason for male infertility seen in Se deficiency (Maiorino, Roveri et al. 2006). The three thioredoxin reductases are also selenoproteins (Mustacich and Powis 2000; Lu, Berndt et al. 2009). These enzymes reduce thioredoxin and help to maintain cellular thiol redox status (Lu, Berndt et al. 2009) .

Other selenoproteins include the family of deiodinases which are involved in thyroid hormone metabolism (Bianco and Larsen 2006). There are three iodothyronine deiodinases. Types I and II help for conversion of T_4 to T_3, while the Type III enzyme

inactivates T_3 (Bianco and Larsen 2006). Selenophosphate synthetase 2 (SPS2), is also a selenoprotein which synthesizes the Se donor for SeCys biosynthesis (Low, Harney et al. 1995; Guimarães, Peterson et al. 1996). Other types of selenoproteins which participate in defending against oxidation include selenoprotein-W, selenoprotein-P and methionine sulfoxide reductase. Selenoprotein P also serves to transport Se to peripheral tissues (Burk and Hill 2005)

Selenium Concentration in the Body

The total Se content of the human body is estimated to range from 13-23 mg, based on cadaver studies (Sunde 2000). Using stable isotope methodology, in US subjects, total body Se was estimated to reach 30 mg (Swanson, Patterson et al. 1991). About 61% of Se is stored in muscle, liver, blood, and kidneys and this proportion rises to 91% if the skeletal system is included (Sunde 2000). On the other hand, cells that have higher concentration of Se are the immune cells, erythrocytes and platelets (Sunde 2000). Normal levels of Se in the body are reported to be 0.1– 0.34 mg/L (1.27–4.32 μmol/L) for white blood cells; 0.04–0.6 mg/L (0.51–7.6 μmol/L) in serum; 0.03 mg/L (<0.38 μmol/L) in urine and < 0.4 μg/g (0.01 μmol/L) in hair (Ogra, Ishiwata et al. 2004). The National Health and Nutrition Examination Survey (NHANES) III for US young adults 19-30 years of age reported the mean serum Se concentration to be 127 and 124 μg/L for males and females respectively (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000). The European adults from different countries have different values (Rayman 2000) which ranged from 86 μg/L in Sweden, France, and Italy to 43 μg/L in Serbia. Values for adults in New Zealand are reported to

range from 62- 69 µg/L (Chen, Yang et al. 1980). Individuals in low Se areas like in China have plasma Se concentration of 11–16 µg/L.

Excretion of Selenium

After Se is absorbed from the intestine, it is excreted mainly into urine (Sunde 2000). Dietary intake influences the amount of Se excreted in the urine when consumed at physiological doses. If dietary intake is excessive, Se tends to be exhaled out into breath in addition to the urinary route (McConnell and Roth 1966). Before excretion, Se is methylated sequentially to produce monomethylated Se and trimethylselenonium as urinary and dimethylselenide as expiratory metabolites (Sunde 2000). The concentrations of the two urinary metabolites differ by the Se intake: at lower dietary Se intake, monomethylated Se is mostly excreted while at high level of dietary Se intake, the trimethylated form is predominantly excreted (Itoh and Suzuki 1997). The monomethylated Se in urine is now characterized to be a selenosugar (Se-methyl-N-acetylgalactosamine) (Fig.6) (Kobayashi, Ogra et al. 2002). Overall, Se is regulated at physiological levels by urinary excretion unlike by absorption for other trace elements like iron.

Physiological Functions of Selenium

Se is present in the host as selenoenzymes and selenoproteins, as discussed above. The detailed functions of these enzymes and proteins, thus, characterize the functions of Se as presented below.

GSH-Px

GSH-Px was described, in 1973, as the first selenoprotein with clear metabolic functions (Mills 1957; Rotruck, Pope et al. 1973). The GSH-Px enzymes are classified

into three forms namely cytosolic, phospholipid and extracellular glutathione peroxidases (c-GSH-Px, p-GSH-Px and e-GSH-Px) and have differences in structural, kinetic, immunological and electrophoretic properties (Maddipati and Marnett 1987; Jotti, Maiorino et al. 1994).

The GSH-Px enzymes are known as antioxidants and play a major role in protecting cells and tissues from damage by free radicals. This function makes GSH-Px indispensable for survival of cells (Michiels, Raes et al. 1994). Both intracellular and extracellular GSH-Pxs are effective in reducing hydrogen peroxide and other organic hydroperoxides to prevent injury to cell membranes (Combs and Combs 1984). The GSH-Px has been used as a major indicator of Se status at physiological doses. The justification for using GSH-Px as a marker for Se is related to the linear relationship between whole blood GSH-Px and plasma Se when the concentration of Se is below 100 µg/L (Rea, Thomson et al. 1979). When Se is depleted at experimental and clinical levels the plasma GSH-Px activity is reduced in humans and small animals. In addition, both experimental and clinical Se depletion have been shown to reduce tissue, blood, and plasma GSH-Px activity in both humans and rats (Takahashi, Newburger et al. 1986; Avissar, Whitin et al. 1989). The different GSH-Pxs are also known by specific numbers and the most well characterized ones are presented below.

GSH-Px-1: This enzyme is abundantly found in all tissues and contains about 50% of the body Se (Esworthy, Ho et al. 1997). The SeCys is the active moiety of GSH-Px enzyme and (Chambers, Frampton et al. 1986). GSHPx-1 is necessary for the detoxification of hydrogen peroxide and other hydroperoxides in the host. Research on GSH-Px knockout mice showed increased risk of being affected by peroxides generated

from paraquat toxicity and increased virulence of coxsackie virus in infected mice (Cheng, Ho et al. 1998; Beck, Handy et al. 2004), indicating the role of this enzyme in detoxifying ROS that led to increased susceptibility to toxins and pathogens.

GSH-Px-2: This enzyme was first identified from human liver DNA but later obviously functions in preventing the damage to the intestine from external peroxides.

GSH-Px-3: This enzyme is excreted by kidney cells in humans and accounts for 20% of selenoproteins (Burk, Early et al. 1998). It is also predominantly available in milk and may be the source of Se in the milk (Avissar, Slemmon et al. 1991). The high level of this enzyme in the kidneys seems to be for protecting kidney cells from damage by ROS, as an excretory organ.

GSH-Px-4: Is commonly found in sperm and testis (Ursini, Heim et al. 1999). The reason may be due to the need to reduce the high level of hydroperoxides being generated during spermatogenesis as a substitute for glutathione in case of shortage (Sunde 2000). More so, it is believed to be part of the structural protein of the sperm to maintain integrity. Deficiency of this enzyme was found to be associated with increased breakage of sperm mid-piece leading to male infertility (Yant, Ran et al. 2003).

Selenoprotein P

Selenoprotein P was first recognized in the plasma of rats and constituted about 50- 60 % of plasma Se (Burk and Hill 2005). Selenoprotein P is secreted by the liver (Burk and Hill 2005). Patients with liver disease tend to have decreased plasma concentration of selenoprotein P (Sunde 2000). During Se deficiency, the level of selenoprotein P is reduced to about 5-10% of the control level (Persson-Moschos, Huang et al. 1995), which signifies the importance of dietary Se in regulating selenoprotein P.

Synthesis of selenoprotein P is given a higher rate of priority compared to other selenoproteins and declines less rapidly than GSH-Px when the exogenous Se supply is limited (Burk and Hill 1993). There is a suggestion that selenoprotein P can be used as a marker for Se in individuals with adequate Se intake as the level of senenoprotein P correlates with plasma Se level (Persson-Moschos, Huang et al. 1995). Selenoprotein P also serves to transport Se in blood (Motsenbocker and Tappel 1982). Selenoprotein P gene knockout mice showed decreased level of Se in testis and brain with increased level of urinary Se excretion which seems consistent with the transport role of the selenoproteins P.

Selenoprotein W

Selenoprotein W is predominant in the muscle and is believed to have antioxidant function as it binds with glutathione, though its functions are not well characterized (Beilstein, Vendeland et al. 1996). The discovery of selenoprotein W came from the investigation of the factor involved as the cause of white muscle disease in Se deficient sheep as deficiency of this factor led to the disease (Beilstein, Vendeland et al. 1996).

Selenophosphate Synthetase-2

Selenophosphate synthetase (SPS2) is a selenocysteine containing selenoprotein that plays a role in providing active Se for the synthesis of SeCys in mammals**.** SPS2 is essential for selenoprotein biosynthesis (Xu, Carlson et al. 2007).

Iodothyronine Deiodinases

Iodothyronine deiodinases are required for metabolism of thyroid hormones (Larsen and Berry 1995). Thyroxine 5'- deiodinase-1 (DI-1), or Type 1, is abundantly present in liver and converts thyroxin (T_4) to triiodothyronine (T_3) that circulates in

plasma (Larsen and Berry 1995). When Se is deficient, the activity of DI-1 decreases and results in lower level of circulating T_3 (Larsen and Berry 1995). Deiodinases Type II and Type III (DI-2 and DI-3) are present in different types of tissues of the body. DI-2 and DI-3 are present in brain, pituitary, brown adipose tissue, placenta and skin with major roles of producing T_3 in these tissues (Sunde 1997). DI-3 plays a role in deiodination of T_4 and T_3 into inactive forms, thus avoiding high levels of T_3 and T_4 in the body (Sunde 1997).

Thioredoxin Reductase (TR)

Mammalian TRs are selenoenzymes which catalyze reduction of intracellular molecules thereby contributing to antioxidant defense systems in the cells (Sun, Wu et al. 1999). TR-1 is located in the cytosol and nucleus, while TR-2 is present in the mitochondria (Sunde 1997). When Se is deficient in rats, the TR activity is less affected than GSHPx-1 activity but more affected than selenoportein P (Sunde 1997). The discovery that elucidated the role of TR in reducing vitamin E and dehydroascorbate to the ascorbate radical further substantiated the ways by which Se serves as an antioxidant (May, Qu et al. 1998) and perhaps as an anticarcinogen also.

The Relationship of Se with Selenoproteins

The selenoproteins represent the largest portion of body Se and are regulated by the SeCys pool (Sunde 1997). The effect of Se level on selenoprotein function was studied in rats and showed differential expression of selenoproteins based on the body's Se status (Lei, Evenson et al. 1995). Liver GSH-Px-1 activity in Se deficient male rats showed a decrease of 1-7 % compared to Se adequate animals (Lei, Evenson et al. 1995).

Severity of Se deficiency leads to significantly lower levels of mRNA for GSH-Px -1 and of GSH-Px-1 protein (Hatfield, Berry et al. 2006) .

When weanling rats fed Se deficient diet were supplemented with graded dietary Se, the liver GSH-Px-1 and its mRNA showed a sigmoid response with increased level of dietary Se intake (Sunde, Evenson et al. 2005). The study showed, when Se intake is higher than 0.1µg Se/g diet, the Se status fails to regulate both GSH-Px-1 activity and its mRNA. In contrast, the liver GSH-Px-4 activity decreased only to 40% of the Se adequate level and reached a plateau at 0.05 µg Se/g diet while the mRNA for liver GSH-Px-4 remained not significantly affected by Se intake (Sunde, Evenson et al. 2005). The activity of plasma GSH-Px-3 was also reduced in these deficient rats to 7- 8% of the level in Se adequate rats and reached to a plateau at 0.07µg Se/g diet (Sunde, Evenson et al. 2005). Other studies also demonstrated that liver TR, DI-1 and selenoprotein P activities in Se deficiency decreased to 5-10% of the Se adequate level (Hadley and Sunde 200*1*).

In sum, these studies show obvious differences in level of selenoproteins by Se status. When Se is deficient, there will be reduced synthesis of protein, leading to decreased levels of selenoproteins (Sunde 1997). Factors other than Se deficiency such as age, pregnancy, lactation, and gender may also affect transcription of selenoproteins and these factors need to be considered when Se status is evaluated using selenoproteins (Sunde, Evenson et al. 2005). Considering the progress in the sequencing of the human genome and the current scientific advancement, mRNA evaluation of selenoproteins might be the preferred approach in evaluating Se status in the future.

Effect of Selenium on Immune Response

Modulation of the immune system by Se involves various processes in the immune system and the following mechanisms have been identified as the most probable ways by which Se affects the immune system (McKenzie, Beckett et al. 2006).

1) Detoxification of excessive ROS such as organic hydroperoxides and hydrogen peroxide;

2) Regulation of eicosanoid synthesis pathways, which leads to favorable synthesis of leukotriene (pro-inflammatory property) and prostacyclin (prevents platelet aggregation) over thromboxane (promotes platelet aggregation) and prostaglandins (immune suppressant);

3) Decreasing expression of pro inflammatory cytokines and adhesion molecules, and,

4) Increasing IL-2 receptor expression for enhanced activities by lymphocytes, natural killer and lymphokine activated killer cells.

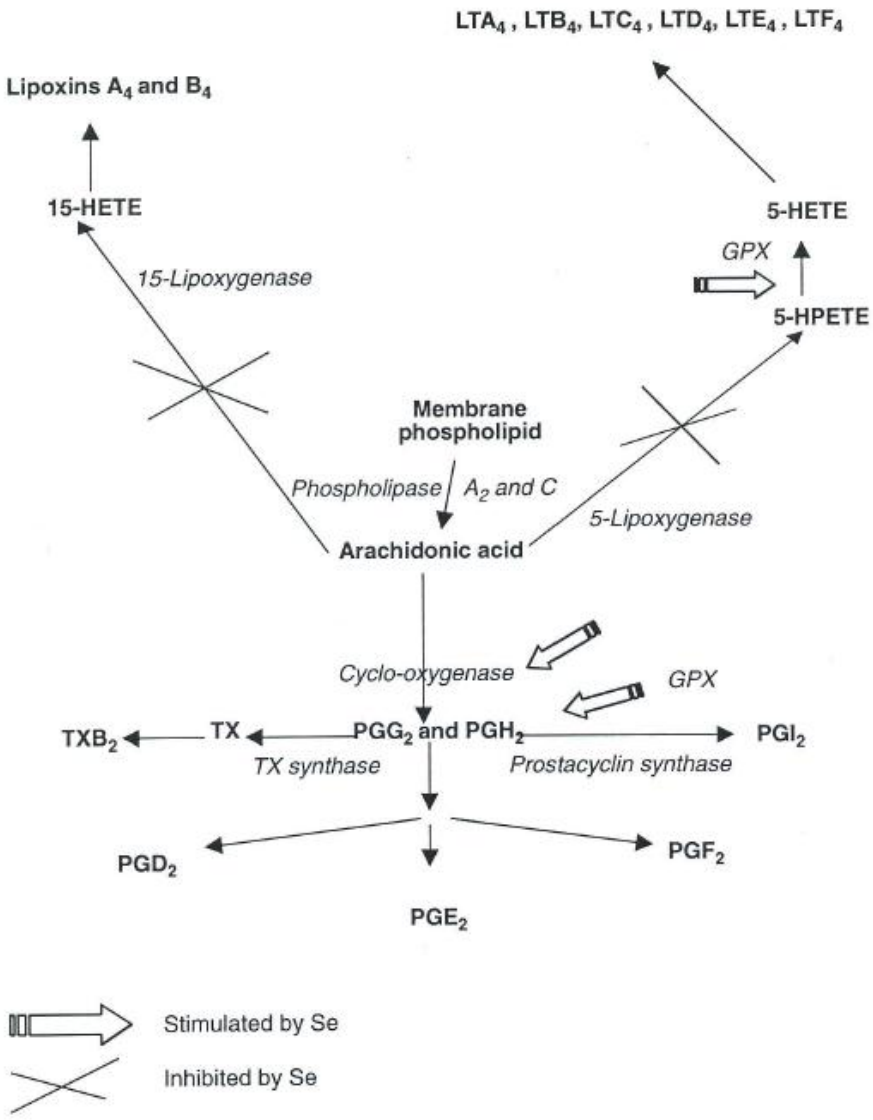
The Function of Selenoenzymes as Peroxynitrite Reductases

Guarding cells against oxidative damage is likely one of the mechanisms by which Se modulates immune response in the host (Sies, Klotz et al. 2002). Under normal circumstances, the innate phagocytic cells produce nitric oxide (NO) which serves as a microbicidial agent. However, in an oxidative environment, the superoxide produced by neutrophils and phagocytes reacts with NO and produces peroxynitrite (ONOO) which is toxic to tissues and damages DNA (Sies, Klotz et al. 2002). Several *in vitro* experiments have demonstrated the value of selenocysteine and selenomethionine in the cell culture to prevent plasmid DNA from being damaged by ONOO (Sies, Klotz et al. 2002).

The Role of Se in Eicosanoid Metabolism

The metabolites of arachidonic acid such as leukotrienes, thromboxane, prostaglandins and lipoxin are considered as eicosanoids (Yu-Zhang, Reddy et al. 2000). Se as part of GSH-Px, modulates eicosanoid metabolism. (Sies, Klotz et al. 2002). Se most obviously seems to exert anti-inflammatory effects as it blocks the release of inflammatory mediators, organoperoxides, which support the secretion of leukotrienes (Fig.2.3) (d'Alessio, Moutet et al. 1998). The capacity of selenoenzymes for blocking 5- and 15- lipoxygenase enzymes, which convert arachidonic acid to the 5-hydroperoxyeicosatetraenoic acid (precursor of leukotriene) is considered an anti-inflammatory function of Se (Tolando, Jovanović et al. 2000). In addition, TR, which is one of the selenoenzymes, helps to convert selenite to selenide which reduces availability of selenite to block the activity of lipoxygenase (Tolando, Jovanović et al. 2000). This adds to the anti-inflammatory function of Se (d'Alessio, Moutet et al. 1998). On the other hand when Se is deficient, it leads to reduced levels of leukotriene B_4 which impairs functions of phagocytes (Tolando, Jovanović et al. 2000). This may lead to decreased levels of phagocytosis causing an increase in virulence of pathogens as a result of weakened first line immune system.

Fig. 2.3: The Effects of Selenium on the Production of Eicosanoids (Calder, Field et al. 2002)



Impact of Se on Adhesion Molecules and Cytokines

Increased levels of inflammatory cytokines induce production of adhesion molecules (d'Alessio, Moutet et al. 1998). Among the cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) stimulate up-regulation of the adhesion molecules as demonstrated in an inflammatory condition (d'Alessio, Moutet et al. 1998). Evidence consistently shows that Se deficiency up-regulates expression of adhesion molecules through regulation of pro-inflammatory cytokines (Tolando, Jovanović et al. 2000). Se deficient cells show higher levels of constitutive expression of adhesion molecules (Tolando, Jovanović et al. 2000). On the other hand, Se supplementation of deficient cells decreases the expression of adhesion molecules (d'Alessio, Moutet et al. 1998). For example, a study on human endothelial cells with a GSH-Px mimic showed reduced expression of intercellular adhesion molecule-1 and vascular adhesion molecule-1 (d'Alessio, Moutet et al. 1998). Use of GSH-Px analogs also prevented expression of P-selectin, E-selectin and release of IL-8 stimulated by TNFα and IL-1 in these cells (d'Alessio, Moutet et al. 1998). Such functions of Se are beneficial to down-regulate excessive inflammatory response in order to minimize tissue and organ damage if uncontrolled.

Effects of Se on Humoral and Cell-mediated Immunity

Se deficient rats showed a slight decrease in the production of IgG fractions, but with no effect on production of IgA (Bauersachs, Kirchgessner et al. 1993). However, production of IgM was markedly lowered and was even more lowered by vitamin E deficiency (Bauersachs, Kirchgessner et al. 1993) and partial improvement of IgA and IgM production resulted from Se supplementation (Bauersachs, Kirchgessner et al. 1993).

Supplementation of Se at 120 µg/kg diet to cows showed increased levels of IgG in cows and their calves (Finch and Turner 1996). Poultry fed on a Se rich diet (1ppm) also demonstrated improved antibody response to salmonella and aflatoxin vaccination (Hegazy and Adachi 2000) supporting the role of Se in immune defense.

Se supplementation also improved cell mediated immune response in many studies. For example, candidacidal activity of neutrophils was lowered and survival against staphylococcus aureus infection was reduced in Se deficient rats (Boyne, Arthur et al. 1986). After 64 days post infection of mice with parasite Trypanosoma cruzi and supplementation with different doses of Se (at 0 ppm, 2 ppm, 4 ppm, 8 ppm, or 16 ppm as sodium selenite) in drinking water (Davis, Brooks et al. 1998) the total death in the Se un-supplemented group of mice was 100% (Davis, Brooks et al. 1998). Of the mice supplemented with 4 and 8 ppm, only sixty percent survived while survival among the group fed with 16 ppm was reported to be 20% (Davis, Brooks et al. 1998). This study supports the role of Se in improving immune response by decreasing the virulence and associated oxidative stress by the pathogen, though 8 ppm and 16 ppm were reported to be toxic doses.

Effects of Se on Interleukin-2 Receptor and Lymphocytes

Se increases the function of both T and B - lymphocytes by up-regulating IL-2 receptor (R) α and β subunits both in mice and humans (Kiremidjian-Schumacher, Roy et al. 1992; Kiremidjian-Schumacher, Roy et al. 1994). Increased high affinity of IL-2 R augments proliferation and differentiation of cytotoxic effector cells (Kiremidjian-Schumacher, Roy et al. 1994). Supplementation of Se in humans (200 µg/day for eight weeks) resulted in an increased activity of cytotoxic T-cells and natural killer cells by

118% and 82% respectively (Kiremidjian-Schumacher, Roy et al. 1994). The activity of suppressor T-cells, however, was down-regulated in these subjects (Kiremidjian-Schumacher, Roy et al. 1994). A study conducted in rats with supplemental doses of selenite in water (0.5 ppm, 2.0 ppm or 5.0 ppm), showed enhanced natural killer cells response in mice supplemented with 0.5 and 2.0 ppm selenite (Koller, Exon et al. 1986) However, the natural killer cell activity in rats supplemented with 5.0 ppm group was identical with that of the un - supplemented group (Koller, Exon et al. 1986). This may be due to immune inhibitory effects of high level Se in mice. The synthesis of antibody showed no significant increase in all groups and was even lower in the group which received 5.0 ppm Se (Koller, Exon et al. 1986). When human lymphocyte cells in culture were treated with a high dose of selenite (0.8 µg/ml), it resulted in an inhibitory effect on natural killer cells activity and lymphokine activated killer cell activities (Nair and Schwartz 1990). This finding was attributed to inhibitory effects of toxic Se concentration (Nair and Schwartz 1990). This study also showed inhibited proliferation of lymphocytes to T-cell mitogens with Se concentration in the range of 0.5-1.0 ug/ml (Nair and Schwartz 1990).

The role of Se in cellular immunity was further elaborated in a study carried out in uremic patients (Bonomini, Forster et al. 1995). The uremic patients had lower plasma Se compared to control groups. The uremic patients were supplemented with 500 µg of Se three times per week for three months followed by 200 µg/day for the next three months (Bonomini, Forster et al. 1995). The result showed increased response to delayed-type hypersensitivity (to phytohemoagglutinin) in the group supplemented with Se after 6 months (Bonomini, Forster et al. 1995; Kiremidjian-Schumacher, Roy et al. 1996)

compared to their baseline and placebo group. This study, however, didn't show any change in lymphocyte number and sub - populations (Bonomini, Forster et al. 1995) .

Requirements of Selenium

Methods used to assess nutrient requirements for other nutrients may be inappropriate to determine Se requirement due to the presence of the unregulated SeMet pool which reflects SeMet intake other than Se status (Sunde 1997). On the other hand, selenoenzyme expression is regulated by Se status and, for this reason, a biochemical approach (instead of dietary intake or tissue concentration or balance studies) was used to determine the RDA for Se intake. In 1980, an initial estimated safe and adequate daily dietary intake was extrapolated for humans (50 to 200 µg Se/d) from animal experiments that assessed Se status using the activity of GSH-Px (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000) .

The Food and Nutrition Board of the US Institute of Medicine (FNB) in 2000 evaluated the level of Se that plateaued the plasma GSH-Px-3 for Chinese men and adjusted the requirement for North American males to 52 µg Se/d (Table. 2.1). The data from New Zealand was evaluated by the FNB and the plasma GSH-Px activity increase between the group who consumed 38 µg Se/d was found to be not different from the group who consumed 68 µg Se/d and the Estimated Average Intake (EAR) was suggested to be 38 µg Se/d. The Adequate Intake for Se varies according to age (Table.2.1). Based on level of Se concentration in breast milk, 15 and 20 µg Se/d is calculated for under six months and 6-12 months old infants respectively. The RDA during pregnancy is 60µg Se/d based on fetal transfer and Se excretion in milk (Table 2.1). The recommendations

for Se intake in the rest of the world are lower than the United States of America which recommends 55 µg Se/d (Table 2.1).

Table.2.1: Selenium Intake for Healthy US and Canadian Populations (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000)

Age group in years	0-6 months	7-12 months	1-3	4-8	9-13	14-18	19-30	31-50	51-70	>70	Pregnancy	Lactation
AI µg/day	15	20										
RDA µg/day			20	30	40	55	55	55	55	55	60	70

AI: Adequate Intake; RDA: Recommended Dietary Allowance

The World Health Organization (WHO) recommended Se intake based on Se needed to achieve two-thirds of maximum achievable GSHPx-3 activity (World Health Organization 1996). With adjustment for inter-individual variations, 40 µg/d and 30 µg/d were proposed for adult males and females respectively which is in line with typical Se consumption worldwide (Table.2.2) (World Health Organization 1996). The New Zealand study used 67% of maximum GSH-Px-3 activity and calculated Se intake of 39 µg/d which was similar to what WHO recommended.

Table.2.2: Recommended Nutrient Intake of Selenium (µg/day) (FAO/WHO 2001)

Age Group	Assumed Weight	Average normative requirement $Se_R^{normative}$ (kg/day)	Average normative requirement $Se_R^{normative}$ (total/day)	RNI[c], µg/day
Infants and children				
0–6 months	6	0.85	5.1	6
7–12 months	9	0.91	8.2	10
1–3 years	12	1.13	13.6	17
4–6 years	19	0.92	17.5	22
7–9 years	25	0.68	17.0	21
Adolescents				
Female,10–18 years	49	0.42	20.6	26
Male, 10–18 years	51	0.50	22.5	32
Adults				
Female,19–65 years	55	0.37	20.4	26
Male, 19–65 years	65	0.42	27.3	34
Female, 65+ years	54	0.37	20.2	25
Male, 65+ years	64	0.41	26.2	33
Pregnancy				
2nd trimester				28
3rd trimester				30
Lactation				
0–6 months post-partum				35
7–12 months post-partum				42

[a] Weight (kg) interpolated from FAO/WHO (reference *86*, page 8, Table 2.1).
[b] Derived from WHO-FAO-IAEA values (reference *86*, page 116, Table 6.1, by interpolation).
[c] Recommended nutrient intake (RNI) derived from average $Se_R^{normative}$ + 2 x assumed standard error (of 12.5 percent)

Dietary Sources of Selenium

Se content of food varies depending on the concentration of Se in the soil. The concentration of Se in cereals and grains varies from < 0.1 to > 0.8 µg Se/g and fruits and vegetables typically have < 0.1µg Se/g (World Health Organization 1996). Foods grown in areas where Se is deficient have much lower levels of Se/g compared to food grown in seleniferous areas (World Health Organization 1996). Se content of livestock also depends on Se content of the food they consume. Concentration of Se in organ meats and sea foods ranges from 0.4 to 1.5 µg Se/g. Muscle meats contain 0.1 to 0.4 µg Se/g and dairy products contain 0.1 to 0.3 µg Se/g (World Health Organization 1996). In the United States most livestock are supplemented with inorganic Se and animal foods have higher levels of Se as selenoproteins (World Health Organization 1996). Generally,

drinking water has insignificant amount of Se, but well water in seleniferous areas may contain higher Se content.

Selenium Toxicity

Se was known for its toxicity in early days due to its manifestations in livestock. This was attributed to high levels of Se in soil and plants(Subcommittee on Laboratory Animal Nutrition. National Research Council 1995). The minimum dietary requirement for rats is said to be 0.1 µg Se/g diet (Sunde 2006). Dietary concentration above 2 µg Se/g diet are considered toxic as it is 20 times more than the minimum requirement (Sunde 2006). Inorganic Se and selenoaminoacids have increased bioavailability and could be toxic, if consumed in excess, as opposed to methylated forms (trimethylselenonium chloride, dimethylselenide) which are less toxic. The hydrogen selenide is the most toxic form compared to all other forms of Se. The body doesn't have a homeostatic mechanism to decrease Se absorption even under chronic toxic intake (Hatfield 2001). In South Dakota and Wyoming of the United States, a study revealed that Se intake was as high as 724 µg/d with no evidence of toxicity (Sunde 2006). Se intake lower than 800 µg Se/d in humans has not been found to be toxic (Goldhaber 2003). Higher levels (50 times higher than the standard 10µg/L) of inorganic Se in well water resulted in increased Se in urine in humans but not in blood (Valentine, Faraji et al. 1988). Blood Se concentration in this study did not reflect the exposure to increased Se intake (Valentine, Faraji et al. 1988).

Chronic Se toxicity is much more common than acute toxicity. Se intake in grams leads to severe gastrointestinal and neurological problems, renal failure, myocardial infarction and respiratory distress (Sunde 2006). Selenosis in humans is associated with

altered nail structure and loss of nail and hair (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000). An average consumption of 1.26 mg Se/d also leads to changes in structure of finger nails as a measure of chronic exposure to high Se intake (Yang and Wang 1994). When Se is consumed at 3.2 mg to 6.7 mg/d for longer periods it leads to lesions in gastrointestinal and nervous system (Sunde 1997). Based on studies in seleniferous regions in China (Yang and Wang 1994), FNB proposed a no-observed adverse effect level (NOAEL) of 800 µg Se/d .

Health Consequences of Selenium Deficiency

Manifestations of Se deficiency are species specific. In mouse, deficiency causes degeneration of muscle and organs such as liver and pancreas and reproductive failure in male rodents due to defects in sperm production (Sunde 2006). When rats were fed Se, vitamin E and sulfur amino acid deficient diets it caused liver necrosis (Subcommittee on Laboratory Animal Nutrition. National Research Council 1995), which may be due to excessive tissue damage from free radicals. Se deficiency in swine resulted in cardiac problems characterized by mulberry heart, while deficiency produced white muscle disease in lambs and gizzard myopathy in turkeys (Panel on Dietary Antioxidants and Related Compounds. Food and Nutrition Board. Institute of Medicine 2000) . In cattle, Se deficiency resulted in muscle myopathy and reproductive system problems, manifested as reproductive failure in bulls and retention of placenta in cows (Sunde 2006). Chickens with severe Se deficiency manifested with symptoms related to exudative diathesis secondary to degeneration of capillary beds (Sunde 2006) . The reasons for these species specific manifestations of Se deficiency are not clear.

In humans, Keshan disease is characterized as the major Se deficiency disease in children and is manifested as cardiomyopathy and occurred in China where Se deficiency was prevalent (Li, Wang et al. 1985). This disease was also compounded with an infection from a virulent coxsackie virus when Se was deficient in the host (Chen, Yang et al. 1980). Coxsackie virus has been isolated from Keshan disease patients suggesting that Se influences the degree to which the pathogen could be virulent.

Kashin-Beck disease was also identified in areas where Se was severely deficient. It is a disease of cartilage and is common in preadolescents and adolescent children (Yang, Ge et al. 1988). Se supplementation, however, didn't avert the disease condition indicating coexistence of other mineral deficiencies that may play roles in the causation of Kashin-Beck disease (Moreno-Reyes, Mathieu et al. 2003).

Patients on total parenteral nutrition without Se supplement are prone to Se deficiency based on a study report from New Zealand on a patient that underwent total parenteral nutrition (van Rij, Thomson et al. 1979). The patient developed dry flaky skin and bilateral muscular myalgia and pain with a great drop of plasma Se from 25 µg/L to 9µg/L after surgery and TPN. Similarly, in the US, muscle pain and cardiomyopathy were reported in patients receiving TPN (Sunde 2006). These patients were found to have low plasma Se, GSH-Px-1 activity and high markers of tissue damage (Sunde 2006) .

Selenium and iodine deficiency interact and lead to the development of endemic myxedematous cretinism manifested by goiter and lowered intelligence and neurological disorders (Sunde 2006). Se supplementation alone leads to aggravation of the disease due to activation of deiodinases, which increased synthesis of thyroid hormone causing

further iodine deficiency, if iodine is not supplemented (Vanderpas, Contempre et al. 1993).

Interaction of Selenium with other Nutrients

Se interacts with other nutrients that affect cellular redox status. Nutrients which play roles in the antioxidant system are several. For example, copper and zinc are part of superoxide dismutase and iron is a component of catalase (Hatfield 2001). Se also interacts with vitamin E in minimizing lipid peroxidation (Sword, Pope et al. 1991) and with vitamin C as TR catalyzes regeneration of the reduced form of vitamin C from its oxidized form, dehydroascorbic acid (Burk 2002).

The role of Se in iodine metabolism makes it an important nutrient in thyroid hormone synthesis and this shows significant interactions between these nutrients (Sunde 1997). The effect of iodine deficiency is exacerbated with concomitant Se deficiency. Se dependent enzymes iodothyronine deiodenases are important for conversion of T_4 to its biologically active form of T_3 (Bianco and Larsen 2006).

Methods of Selenium Assessment

The possible roles of selenoproteins as biomarkers of Se status are under investigation. Of these proteins, iodothyronine deiodinase seems to have a potential role as a biomarker for thyroid hormone metabolism taking the T4:T3 ratio. At the same time this marker could be utilized as a functional marker of Se status (Gibson 2005). Investigations also showed that selenoproteins were found not to respond equally to changes in Se status (Arthur 1999). Arthur suggested possible markers that could indicate Se status and these are plasma or whole blood Se concentration, plasma GSH-Px-3 activities, erythrocyte GSH-Px-1 activities, selenoperoxidase activities, plasma selenoprotein P and thyroid hormone levels as discussed above.

Plasma Se

Protein bound Se is associated to α and β - globulins of lipoproteins. Plasma and serum Se concentrations are comparable and both reflect short term changes in Se intake, mainly of SeMet compared to inorganic forms of Se (Levander, Sutherland et al. 1981). SeMet is not subject to homeostatic control as this form of Se incorporates into tissue proteins in place of methionine (Burk and Levander 1999). Plasma Se values less than 0.1µmol/L are associated with depletion and with clinical features of deficiency (Tereda, Nakada et al. 1996). There are no universally agreed upon cut-off values for plasma Se (Gibson 2005). Cut-off points suggested by Thomson is only for assessment of the adequacy of Se (Thomson 2004). Plasma or serum Se are measured more accurately using inductively coupled plasma mass spectrometry (ICP-MS) (Gibson 2005). Plasma Se is said to be affected by Se intake, age, puberty, pregnancy and lactation, prematurity, smoking and chronic diseases in humans (Gibson 2005). Due consideration must be given to these factors while interpreting results.

Whole Blood Se

Whole blood Se is stable and is used as an index of long term Se intake (Gibson 2005). The whole blood Se changes after a period of depletion (months), which makes the relationship of current Se intake with whole blood concentration a bit difficult to associate (Gibson 2005). As a result, criteria for interpretation of the values of whole blood Se have not yet been established. Whole blood Se could also be assessed using AAS and ICP-MS, though the analysis is said to be difficult. Factors affecting plasma Se affect concentration of Se in whole blood as well (Gibson 2005)

Erythrocytes and Platelet Se

Erythrocyte Se is mostly related with the hemoglobin, while only 15% is associated with its glutathione peroxidase. This too reflects long term Se status. For people consuming stable intakes of Se, positive correlation was seen between erythrocytes, plasma and dietary intake (Lane, Dudrick et al. 1981) Erythrocyte Se is lower in disease conditions that affect absorption of Se and it responds slowly to Se supplementation compared to plasma Se. The longer period required for the synthesis of erythrocyte and the limited transferability of hemoglobin-bound Se contributed to slow response of erythrocytes to Se supplementation (Nève 1995). The type of Se used for supplementation determines the rate of response by erythrocytes. The erythrocytes response to supplementation with inorganic Se is slower than SeMet, even though SeMet is not subject to homeostatic regulation (Thomson, Ong et al. 1985). Determination of erythrocyte Se is not highly recommended due to problems with measurements. Information on factors affecting erythrocyte Se concentration is lacking but associations exist between chronic diseases affecting Se absorption, long term low Se intake, genetic diseases such as sickle cell anemia and Down's syndrome (Neve 1999)

Urinary Se

Se excretion in urine helps to regulate homeostasis of Se in the body and it is the major excretory pathway for Se (50 - 60%), while the remaining gets excreted via feces (Levander and Burk 1994). Urinary excretion correlates well with dietary intake and plasma Se such that dietary Se intake can be roughly estimated to equal twice as much as urinary Se (Thomas 1998). Urinary Se excretion is lower in females, pregnant women and aged people (Gibson 2005) and reduction of Se in the aged population is associated with reduction in muscle mass (Glover 1967). Urinary Se is used as an index of toxicity

and the allowable maximum concentration is at 1.3 µmol/L (Hojo 1981). Fasting urine samples are preferred for measurement of urinary Se at the population level (Gibson 2005). Fluorometric method is commonly used but the AAS method can also be used (Gibson 2005).

GSH-Px

Se status could be assessed through the measurement of individual selenoproteins in blood (Gibson 2005). GSH-Px-1 activity in erythrocytes is used to assess Se status in individuals when Se intake is below the threshold (1.15 µmol/L) and it correlates with whole blood or erythrocyte Se level (Duffield, Thomson et al. 1999). This would mean that the activity GSH-Px-1 is dependent on Se status but when Se concentration is above "threshold" value, correlation doesn't exist and this makes it complicated to use it as marker of Se status (Gibson 2005). GSH-Px-1 activity in platelets is also considered as a sensitive indicator as platelets contain significantly higher concentration of Se than any other tissues (Gibson 2005). However, separation of platelets is difficult, even though the response to Se supplementation is found to be faster, due to their rapid - turnover.

GSH-Px-3 contains 12% of the Se in plasma (Xia, Hill et al. 1989). GSH-Px-3 is measured more accurately than other GSH-Pxs (Xia, Hill et al. 1989). Strong correlation has been identified between plasma Se and GSH-Px-3 activity (Thomson, Ong et al. 1985), and plasma Se and GSH-Px-3 are said to be good measures of Se status (Burk and Levander 1999). Plasma GSH-Px-3 activity increases following supplementation and this is not dependent on the type of Se used for supplementation (Gibson 2005). GSH-Px -3 is also used in population studies where Se status is low, like the erythrocytes and platelets GSH-Px (Lane, Dudrick et al. 1981). GSHPx-3 is more stable at -80C than GSH-Px-1

activity. Enzyme-linked immunosorbant assay (ELISA) kits are also used, provided heparin is used as anticoagulant (Gibson 2005).

Selenoprotein P

Two thirds of plasma Se is present as selenoprotein P. It is present in different tissues and is secreted into plasma by the liver (Burk and Levander 1999). Selenoprotein P is said to be more sensitive to Se deficiency than plasma GSH-Px-3 activity (Duffield, Thomson et al. 1999). Response to Se supplementation by selenoprotein P is higher than GSH-Px-3 (Chen, Yang et al. 1980) and selenoprotein P and plasma Se correlates positively with Se status (Gibson 2005). Optimal level for plasma selenoprotein P is yet to be defined (Gibson 2005). Selenoprotein P could be measured by competitive radioimmunoassay using ^{75}Se labeled human selenoprotein P (Xia, Hill et al. 1989).

Hair and Toe Nail Se

Hair Se is found to have a relationship with whole blood Se (Yang, Wang et al. 1983) and hair also shows a positive response to Se supplementation (Gibson 2005). Elevated hair Se concentration is found in areas with overexposure to Se (Yoshizawa, Willett et al. 1998). Concentration of hair Se can be measured using AAS or instrumental neutron activation analysis (INAA). Concentration of hair Se, however, could be confounded by use of Se containing shampoos. Toenail concentration of Se reflects long term exposure as Se incorporates as the toe nail grows (Gibson 2005). The correlation of toe nail with serum and whole blood Se concentrations makes it a good marker of Se in cohort studies (Yoshizawa, Willett et al. 1998). Methods used for determination of hair Se can be used to measure toe nail concentration of Se as well (Gibson 2005).

Multiple Indices

For individuals with low Se status, the measurement of total Se and GSH-Px-3 in plasma is recommended (Gibson 2005). For those having adequate Se status, Se status could be assessed by total Se in plasma and erythrocytes as a marker of current and longer term status respectively (Gibson 2005). When blood collection is limiting, analysis of toenail Se is recommended as a marker of long term Se status. When Se status is studied as a risk factor for disease, interaction of Se with other antioxidants nutrients, polyunsaturated fats, heavy metals and iodine status must be investigated to rule out any confounding effects of these nutrients (Gibson 2005).

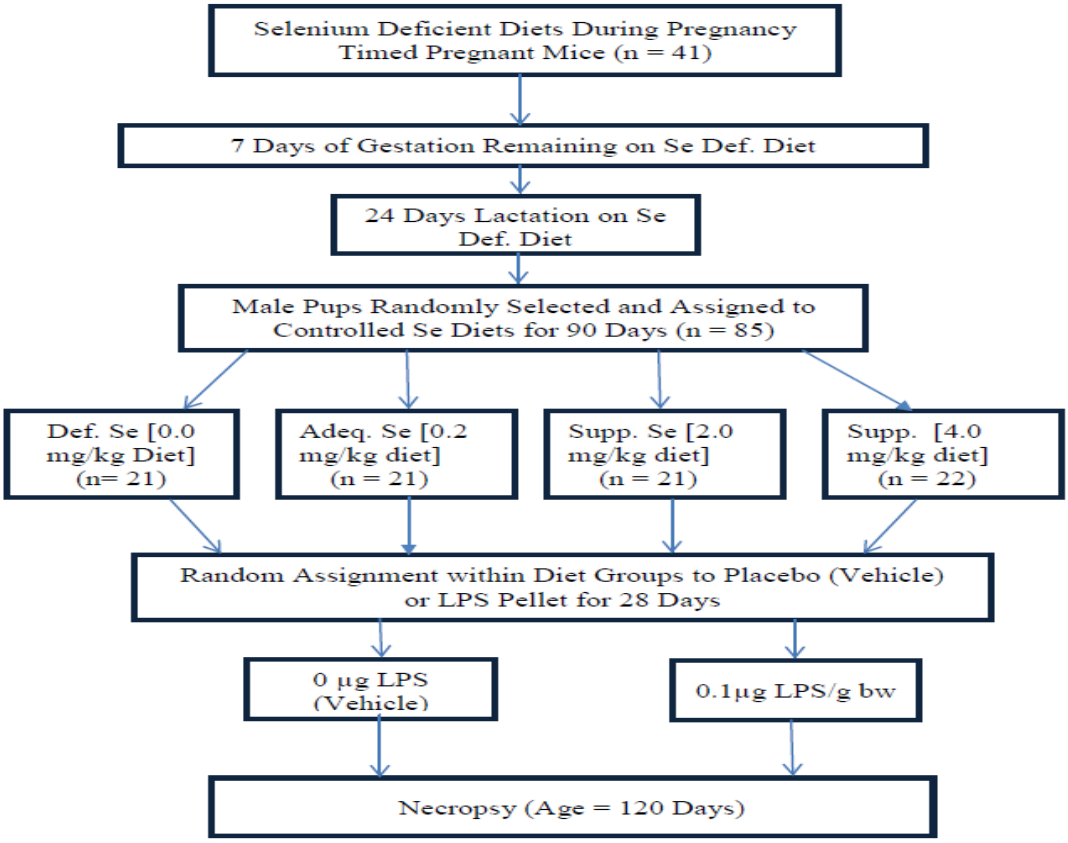
CHAPTER III

METHODOLOGY

Experimental Design and Animals

The study is a 4 x 2 factorial design (four diet groups with LPS and placebo groups) and the following diagram shows the randomization procedure used in the study (Fig. 3.1). The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University (OSU). To demonstrate effects of Se in a relatively short time, second generation selenium-deficient animals were used. Forty-one timed-pregnant C57/BL6 mice (Harlan, Indianapolis, IN) were fed Se-deficient (modified AIN-93G) diets for the final 5-6 days of gestation. Animals were housed in an environmentally controlled animal care facility and delivered their litters approximately 5-6 days after arrival. The dams continued to receive the pre delivery Se-deficient diets for the three weeks of lactation. At 24 days of age, pups were weaned. Weanling male mice were randomly assigned to one of the four dietary treatments and were fed until they reached 120 days of age.

Fig. 3.1: Flowchart of Randomization



Diet was provided daily and water bottles and bedding were changed on a weekly basis. The basal Se depletion diet (Torula yeast-based, approximately 0.02 mg Se/kg diet) was purchased commercially and other diets were prepared commercially or in-house by adding 0.2, 2.0 or 4.0 mg Se/kg of diet as sodium selenate. Mice were fed *ad libitum* (approximately 5g diet/day/mouse). At 90 days of age, mice were randomly assigned within diet groups to implantation of placebo pellet (implantation as a vehicle only) or time-release LPS (E.coli Serotype 0127:B8) pellet to produce an inflammatory stress. The LPS dose was 0.1 µg/g body weight. Treatments remained (time-release LPS or

placebo pellet) for 28 days and mice continued to be fed their respective diets throughout the 28 days until necropsy. Necropsy was carried out at the 28^{th} day post LPS or placebo implantation. At necropsy, mice were anesthetized with ketamine/xylazine at the dose of 0.15 ml/10g mouse weight prior to blood collection and tissue harvesting. All tissues and specimens were properly labeled, packaged and stored at appropriate temperature for analysis.

Necropsy

In preparation for necropsy, necessary surgical instruments and bottles were autoclaved and all preparations for tissue harvests and collection of blood were organized prior to the day of necropsy. Mice allocated for each day of necropsy underwent 12 h fasting. Weight was recorded at 12 h prior to necropsy. On the day of necropsy each mouse was injected with ketamine/xylazine at the dose of 0.15 ml/10g mouse and bone density was measured using Piximus instrument. Blood was collected from carotid artery collection. A drop of whole blood was used to make a blood smear for the WBC differential count. For total white blood cells, a dilution of blood was done by adding 0.025 ml of whole blood to 0.475 ml of diluting fluid (2% acetic acid with 1% crystal violet), mixed and stored at room temperature until counted. The remaining blood was kept in EDTA coated centrifuge tubes on ice for up to 2 -3 hours until the end of necropsy. On completion of necropsy each day, blood was centrifuged for 20 minutes at 4000 rpm and plasma was separated, aliquoted and stored at -80^{0}.

On a sub-sample from each diet group, (six mice per group), the right femur from each mouse was flushed to collect bone marrow. The right femur was lightly crushed and flushed with normal saline using 21 gauge needles and the cell suspension was kept on

ice in conical tubes until processed by addition of fluorescent antibodies for flowcytometer reading.

Laboratory Analysis

Flowcytometry Analysis

The number of bone marrow lymphocytes and total T and B cells population for all mice treated with time - release LPS and fed with different concentrations of dietary Se were compared to dietary equivalent placebo groups. The bone marrow suspension was centrifuged each day after necropsy and cells were then immunostained by adding fluorochrome and conjugated primary antibodies (CD3, B220, CD4, CD31, LY-C6) with appropriate dilution to assay tubes and incubated for 1 hr at room temperature. Cells were rinsed and resuspended with PBS and analyzed using a florescence activated cell sorter (FACS) flow cytometer at a rate of 286 cells per second using a single argon ion laser tuned at 488 nm. Data were analyzed using Summit version 4.3 Build 2445 (Dako Colorado, Inc., Fort Collins, CO). All antibodies mentioned above and reagents were used at final concentrations recommended by manufacturers

Determination of Total and Differential Leucocyte Counts

Determination of the total leucocyte count was made using the method of Schalm *et al.* (Schalm, Jain et al. 1975). The differential white blood cell count was determined from a thin smear done on a clean slide for each mouse. The smear was set to dry and fixed until stained for counting (Wright's stain). Counting was done using an objective light microscope. A longitudinal counting method was used to count 100 cells. The type of cell counted in these 100 cells was used to set percentages for neutrophils, lymphocytes, monocytes, eosinophils and basophils cells. The absolute number for each

type of cell was calculated by multiplying the total WBC counts by the percentage of each cell type.

Plasma Immunoglobulin: IgG2a

Plasma level of IgG2a was assayed using an immunperoxidase Assay (ICL, Inc., Portland, OR). Samples were diluted 1:4000 in two stages. First we mixed 5 µL of samples with 100µL of 1x diluent provided in the kit and vortexed for 10 – 15 seconds. The second dilution was made by mixing 5µl of the first dilution with 1000µl of diluent. Pre-diluted IgG2a calibrator (100µL) was pipetted into a anti-mouse IgG2a ELISA micro plate in duplicate followed by 100 µL of samples in the remaining wells. The plate was incubated for one hour for maximum binding. After the incubation, the solution was decanted and washed four times with wash solution followed by addition of 100µL of pre-diluted Enzyme Antibody Conjugate to each well. The plate was incubated in the dark at room temperature for 20 minutes and then washed and blotted four times. Next 100 µL of 3,3', 5, 5',- Tetramethylbenzidine (TMB) substrate solution was pipetted into each well and incubated in the dark at room temperature for 10 minutes. After 10 minutes 100 µL of stop solution was added to each well and absorbance was determined at 450nm using a microplate reader.

Plasma Cytokine

IFNγ

Plasma IFNγ was measured using an ELISA kit and the protocol provided by the manufacturer (BD Biosciences; San Jose, CA). Pre-diluted standards (50 µL) were pipetted in duplicate followed by samples of the same amount in the remaining wells. The plate was incubated for two hours for maximum binding at room temperature. After

the incubation, the solution was decanted and washed five times with wash solution provided in the kit. Following this, 100 µL of pre diluted detector solution was added to each well and incubated at room temperature for one hour. The plate was then washed and blotted five times. Next 100 µL of TMB substrate solution was pipetted into each well and incubated in the dark at room temperature for 30 minutes. After 30 minutes 50µL of Stop Solution was added to each well and absorbance was determined at 450nm and 570 nm using a calibrated plate reader and change in reading between the two wave lengths was obtained for analysis.

IL-12p70

Plasma IL-12p70 was measured using an ELISA kit with the assay protocol provided by the manufacturer (R&D Systems, Inc., Minneapolis, MN). Pre-diluted assay diluent (50 µL) was pipetted to each well followed by 50µl standard and control in duplicate and samples in the remaining wells. The plate was incubated for two hours for maximum binding at room temperature. After the incubation, the solution was decanted and washed five times with wash solution provided with the kit and blotted. Following this, 100 µL of conjugate was added to each well and the plate was covered and incubated at room temperature for one hour. The plate was then washed and blotted five times. Next, 100 µL of TMB substrate solution was pipetted into each well and the plate was incubated in the dark at room temperature for 30 minutes. After 30 minutes 100µL of stop solution was added to each well and tapped gently for mixing. Absorbance was determined at 540 nm and 570 nm using calibrated plate reader and difference in readings was obtained for analysis.

Plasma Total Antioxidant Capacity (TAC)

Antioxidant assay kit was used to measure TAC in plasma samples (Cayman Chemical Company, Ann Arbor, MI). The assay procedure was done in accordance with the manufacturer's protocol. The assay measured the ability of antioxidants in the plasma samples to inhibit oxidation of 2, 2´-azino-di (3-ethylbenzothiazoline sulphonate) ABTS to ABTS•+ by metmyoglobin. The TAC of samples preventing oxidation of ABTS was compared to the Trolox standard (water soluble tocopherol analog) and quantified as millimolar Trolox equivalents. For the assay, 10 µL of pre-reconstituted Trolox standards, 10µl of metmyoglobin and 150 µl of chromogen were pipetted into a micro-plate followed by 10 µl of samples, 10 µl of metmyoglobin and 150 µl of chromogen in each remaining wells. The reaction was initiated by adding 40 µl of hydrogen peroxide to all wells followed by incubation of the covered plate at room temperature for five minutes on a shaker. Absorbance was read at 405 nm using a calibrated plate reader.

Plasma GSH-Px

Plasma GSH-Px was measured using a kinetic enzyme assay as per the protocol provided by the manufacturer (Oxford Biomedical Research, Inc. Oxford, MI). The assay was carried out at room temperature and the spectrophotometer was set at 340 nm. The spectrophotometer was zeroed at 340 nm using deionized water. Prior to the assay, each sample was diluted 1:10 using assay buffer provided in the kit. An appropriate volume of assay buffer, pre-diluted NADH reagent and sample were pipetted into the cuvette and placed in the spectrophotometer followed by addition of tert-butyl hydroperoxide and mixed by pipetting. The enzyme activity was measured at A340 for three minutes. The GSH-Px coupled reduction of tert-butyl hydroperoxide from the oxidation of NADPH by

glutathione reductase and concomitant oxidation was monitored in a spectrophotometer with the decrease in absorbance at 340 nm. For each reading, the rate of decrease in A 340 /minute was calculated and the net rate for sample was calculated by subtracting the rate from water blank. The net A340/min for each sample was then converted to NADPH consumed. One unit of GSH-Px is expressed as the amount of GSH-Px needed to oxidize 1 μmol of NADPH per min. The value for each sample then was corrected for dilution factors and expressed as GSH-Px uM/mL.

Clinical Analysis

A Biolis 24i clinical chemistry analyzer was used to determine plasma concentrations of ALT, ALP and ALB. Kits were purchased from Carolina Liquid Chemistries Corp. and the manufacturer's instructions were followed.

Statistical Analysis

Statistical analysis was done using SAS version 9.2 (SAS Institute, Cary. NC, USA). Two-way ANOVA was performed using PROC GLM followed by post hoc analysis with Fisher's least significant differences test for means separation when *F* values were significant. Data are presented as mean ± SE and α was set at 0.05.

CHAPTER IV

THE EFFECTS OF DIETARY SELENIUM AND LOW GRADE INFLAMMATION ON SELECTED IMMUNE CELL POPULATIONS IN C57BL/6 MICE

Abstract

Selenium (Se) as a nutrient has many beneficial functions related to its nutritional, biochemical and molecular properties. Se is important for adequate immune response. In this study, dams were fed a Torula yeast-based Se depletion diet for the final week of gestation and throughout lactation. At 24 days of age, pups were weaned to the depletion diet or to diets with 0.2, 2 or 4 mg of added Se (as sodium selenate)/kg of diet. These diets were fed for 14 weeks. Four weeks before necropsy lipopolysaccharide (LPS) time-release pellets (0 or 0.1 μg/g body weight) were implanted subcutaneously. At necropsy, plasma Se was significantly lower in pups fed the depletion diet than in those fed the three Se-supplemented diets. Mean body weight was not significantly different by LPS ($p<0.06$). Interaction affected peripheral differential counts of white blood cells. Mice fed 4 mg Se added/kg diet with LPS showed a significant lower numbers of lymphocytes compared to other groups. Peripheral neutrophil numbers were significantly higher for mice fed 4 mg Se added/kg diet with LPS and lower for mice fed 0.2 mg Se added/kg diet and with placebo.

LPS introduced a significant ($p<0.05$) increase in number of T- helper cells (CD4+)

($0.534x10^6$/mL vs $0.906x10^6$/mL, B-cells (B220+) ($2.53x10^6$/mL vs $3.35x10^9$/L), and monocytes (CD31neg LY-$C6^{hig}$) ($0.765\ x10^6$/mL vs $0.088\ x\ 10^6$/mL) compared to placebo groups. In addition T-cell numbers were greater with increasing Se intake ($p<0.02$). Although the immune response is dependent upon several other factors, our study showed that Se supplementation tended to increase T-cell numbers in response to low grade inflammation induced by LPS. Further understanding of the mechanisms by which dietary Se affects these immune cell populations will contribute to knowledge of using Se supplementation to affect T-cell mediated immune response.

Introduction

The importance of selenium (Se) in human health is well documented (Rayman 2002). The functions of Se are exerted by the selenoproteins and 25 of these have been characterized in humans (Kryukov, Castellano et al. 2003). Most of these selenoproteins, of glutathione peroxidase (GSH-Px) and thioredoxin reductase (TR) are the major ones, have antioxidant functions. Se as part of GSH-Px and TR (Prabhu, Zamamiri-Davis et al. 2002) plays an important role in balancing oxidation reduction (Redox) status in cells when at physiologic doses. Excess Se intake is linked with increased production of reactive oxygen species (ROS) leading to oxidative stress (Yang, Shen et al. 2000; Shen, Yang et al. 2001). Adequate dietary Se regulates nuclear factor kappa beta (NF-κβ) by modulating its effect on mitogen-activated protein kinases (MAPKs) (Park, Park et al. 2000), thus it is involved in stress-induced signaling pathways including inflammatory responses. Based on this information, it is suggested that Se may antagonize inflammatory response via MAPKs signaling. As GSH-Px and TR are expressed by most

cells including immune cells (Bainbridge 1976; Behne and Wolters 1983; Gromer, Eubel et al. 2005), it may be one of the ways by which Se influences the immune response.

Under normal circumstances, the host's immune response to pathogen invasion relies on cell mediated (Type 1) and humoral (Type 2) immunity. Type 1 immune response depends on differentiation of T-helper (Th) cells, (Ho and Glimcher 2002) which are important for both inflammatory and cytotoxic responses. Th-cells activate both macrophages and CD8+ T- cells for pathogen clearing (Ho and Glimcher 2002). The Th- cells are also important for generation of Type 2 immunity through stimulation of the B - cells to produce immunoglobulins (Ho and Glimcher 2002). Type 2 immune response is also very important to neutralize pathogens and contain parasitic infestations (Ho and Glimcher 2002) .

Type 1 and Type 2 immune responses are directed by the two subsets of CD4+ T-helper cells: the Type 1 Th-cell (Th-1) and Type 2 Th-cell (Th-2) (Mosmann and Coffman 1989). Th-1 cells produce IFN-γ, TNF-α and lymphotoxin responsible for delayed type hypersensitivity responses, while Th-2 cells produce IL-4, IL-5 ,IL-10 and IL-13 which enhance B - cell proliferation and allergic responses. INF-γ is the hallmark of Th-1 cytokines, while IL-4 is a marker for Th-2 cells (Ho and Glimcher 2002). The outcome of T- cell differentiation is dependent upon cytokine medium, type of antigen presenting cells, route of antigen presentation and type of stimulatory markers (Ho and Glimcher 2002). The cytokine environment is important in determining differentiation of Th-cells, because cytokines are important for initiation and proliferation of both subsets of Th-cells (Ho and Glimcher 2002). Several nutrients also play roles in the differentiation of T- cells, of which Se is a key nutrient.

At the cellular level, it was demonstrated that Se has effects on different components of the immune system including innate, cell-mediated and humoral responses (Huang, Rose et al. 2011). Cell culture studies showed that Se stimulates immune properties at supra-physiological doses. Deficiency of Se, on the other hand, negatively affects leukocyte proliferation in response to mitogens. This alteration in immune response, as a result of Se deficiency, was evidenced by clinical features such as increased vulnerability to infection and lowered resistance to tumor-related antigens (Peretz, Neve et al. 1991). In our study, mice fed with graded dietary Se were challenged with low dose, timed release lipopolysaccharide (LPS) one month prior to necropsy with the hypothesis that Se would impact low grade inflammation by down-regulating excessive production of pro-inflammatory cytokines and preventing excessive tissue damage.

Stimulants such as free radicals and oxidative stress enhance inflammation by activating nuclear factor-kappa B (NF-κB) pathway (Barnes and Karin 1997). NF-κB is a membrane-bound transcriptional factor that is phosphorylated when induced by ROS and then translocated to the nucleus to up-regulate production of inflammatory cytokines (Barnes and Karin 1997). This cytokine mileau then determines the Th-cell differentiation into the specific sub types of Th-cells. Se down-regulates excess production of pro-inflammatory cytokines. For example, Se supplementation results in a significant decrease in the bacterial endotoxin- induced expression of TNF-α by blocking the mitogen-activated protein kinase (MAPK) pathway (Kim, Johnson et al. 2004). On the other hand, when Se is low, the high level of TNF-α may enhance activation of NF-κB and increase C-reactive protein (CRP) production by liver cells. When Se is adequate,

it prevents NF-κB activation by increasing GSH-Px and this attenuates inflammation by down regulating ROS, which is a stimulator of NF-κB. GSH-Px inhibits translocation of NF-κB by preventing phosphorylation of IκB-α which is an inhibitory subunit bound to NF-κB when inactive (Kretz-Remy and Arrigo 2001).

The biochemical form of glutathione (GSH) in the host also affects the immune response. For example, mice depleted of GSH showed hampered Th-1 response and functions of antigen-presenting cells, which are major players in immune response (Peterson, Herzenberg et al. 1998). When the level of reduced GSH is higher due to increased dietary Se intake (0.8-1.0 mg/kg) it showed Th-1-skewing (up-regulation of IFN-γ secretion) with activation of naive CD4+ T-cells (Hoffmann, Hashimoto et al. 2010). CD4+ T-cell differentiation was not, however, affected in mice with Se intake of 0.25 mg/kg (Hoffmann, Hashimoto et al. 2010). This shows that dietary Se, when present above physiologic level, modulates GSH levels and influences CD4+ cell proliferation and differentiation during inflammation.

Change in the number of B-cells was also observed in mice with Se supplementation. For example, the B-cell number in spleens of mice fed with low level of Se-Met (0.02 mg/kg), adequate (0.2 mg/kg) or high Se (2 mg/kg) in the diet for 50 days showed differences (Vega, Rodríguez-Sosa et al. 2007). Vega and colleagues observed that spleen cells of mice fed low Se-Met had reduced numbers of B-cells when compared to mice fed adequate Se-Met. Mice fed with 2.0 mg Se/kg diet showed increased B- cells in their spleens compared to mice that consumed 0.2 mg Se/kg (control) diet.

Se also exerts its immune enhancing effect by up-regulating IL-2 receptor expressed both by T and B lymphocytes. The response of these cells to IL-2 then results

in increased numbers of lymphocytes, enhanced cytotoxic effect of killer cells and production of immunoglobulin (Ig) by B-cells to respond to inflammatory conditions (Kiremidjian-Schumacher, Roy et al. 1996). The level of Se that is required to exert these immune functions has been variable as reported by Spalholz (Spallholz 1990). In our study we investigated different doses of dietary Se intake on T-cells, Th-cells and B-cells, lymphocytes, granulocytes and monocytes in mice challenged with LPS.

Methods

Mice and diets: Forty-one timed-pregnant C57BL/6 mice (Harlan, Indianapolis, IN) were fed Se-deficient (modified AIN-93G) diets for the final 5-6 days of gestation. Animals were housed in an environmentally controlled animal care facility and delivered their litters approximately 5-6 days after arrival. The dams continued to receive Se depletion diets for the 24 days of lactation. At 24 days of age, pups were weaned and randomly assigned to one of four dietary groups (Table 4.1) which they were fed until 120 days of age. Diet was provided daily and water bottles and bedding on a weekly basis.

Mice were fed *ad libitum* (approximately 5g diet/day). At 90 days of age, mice within each dietary treatment group were randomly assigned to be implanted with a time-release pellet that was a placebo (0 µg/g/d) or that released 0.1 µg/g/body weight of time-release LPS (E.coli Serotype 0127:B8) to produce a very low grade inflammatory stress. Treatment was maintained (time-release LPS or placebo pellet) for 28 days and the mice continued to be fed their respective diets throughout the study.

Collection of Blood Sample from Mice

About 0.5 ml of blood was collected from carotid artery. Out of the total blood collected, 0.25 µL was put into a micro tube with 475 µL blue stain solution for

leukocyte counts. The remaining blood was added to a tube containing anticoagulant and kept on ice until centrifuged at 4,000 revolutions per minute (rpm) for 10 min to separate the plasma for determination of the immune biomarkers.

Determination of Total and Differential Leucocyte Counts

Determination of the total leucocyte count was made using the method of Schalm *et al.* (Schalm, Jain et al. 1975). The differential white blood cell count was determined from a thin smear done on a clean slide for each mouse. The smear was set to dry and fixed until stained for counting (Wright's stain). Counting was done using an objective light microscope. A longitudinal counting method was used to count 100 cells. The type of cell counted in these 100 cells was used to set percentages for neutrophils, lymphocytes, monocytes, eosinophils and basophils cells. The absolute number for each type of cell was calculated by multiplying the total WBC counts by the percentage of each cell type.

Plasma Se

Plasma Se was analyzed using an inductively coupled plasma mass spectrometer (ICP-MS) (Elan 9000, Perkin Elmer, Norwalk, CT). All plasma samples were diluted 50 fold (40 µl diluted with 1.96 ml) with 0.1% HNO_3 (GFS Chemicals, Powell, OH) in deionized water (Milli-Q, Advantage A10, Millipore). Standard solutions of Se were prepared by dilution of certified standard solutions (Perkin Elmer, Norwalk, CT). Diluted working standards were prepared immediately prior to their use by diluting an intermediate stock standard solution. All samples and standards were spiked with 4 µg/L gallium as an internal standard. Quality control samples (Utak Laboratories, Inc.,

Valencia, CA) were utilized in order to verify that performance was within recommended ranges.

Flow Cytometry

Antibodies used for flowcytometric analysis included peridinin chlorophyll protein (PerCP)-anti-CD4 and fluorescein isothiocyanate (FITC)- FITC-anti-CD3, phycoerythrin (PE)-anti-B220, FITC-anti LY6C, and allophycocyanin (APC) - anti CD31 (Sigma-Aldrich Co., St. Louis, MO).

Cell suspension: The bone marrow suspension was centrifuged after necropsy and cells were collected after aspirating supernatant. Cells were resuspended by adding phosphate buffered saline (PBS) and were immunostained by adding fluorochrome conjugated primary antibodies (CD3, B220, CD4, LY6C and CD31) with appropriate dilution to assay tubes and incubating for 1 hr at room temperature. Cells were rinsed and resuspended with PBS and counted using a FACScan flow cytometer at a rate of 286 cells per second using a single argon ion laser tuned at 488 nm. Data were analyzed using Summit version 4.3 Build 2445 (Dako Colarado, Inc., Fort Collins, CO). All antibodies and reagents listed above were used at final concentrations recommended by manufacturers of the flow cytometer.

Plasma IgG2a

Plasma level of IgG2a was assayed using an immunoperoxidase Assay kit (ICL, Inc., Portland, OR). Samples were diluted (1:4000) with 1x diluent provided and all procedures were followed as per the protocol provided by the manufacturer.

Plasma cytokines

Plasma IFNγ was measured using an ELISA protocol provided by the manufacturer (BD Biosciences; San Jose, CA).

Statistical Analysis

All statistical tests for comparison of means were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA). The GLM procedure tested the main effects of dietary Se concentration and of LPS on proliferation of B cells, T-cells, T-helper cells and myeloid populations. A least square means post hoc test was used to identify the means that differed. Differences were considered significant at $p < 0.05$.

Results

Body and thymus weight

There was no statistically significant difference in body and thymus weight by Se intake or LPS at necropsy ($p > 0.05$, Table 4.2), however thymus weight tended to be higher for LPS (0.050g) compared to placebo group (0.043g).

Plasma Se

Plasma Se of mice from different experimental diets at necropsy showed significant differences by diets supplemented with Se (0.0; 0.2; 2.0 and 4.0 mg Se/kg diet) ($p<0.03$). Mice fed the Torula yeast Se-deficient diet with no added Se had significantly lower plasma Se compared with other groups (Table 4.3). However, plasma Se was not significantly affected by LPS.

Total White Blood Cells and Differential Counts

Total white blood cell count was not statistically affected by either dietary Se or LPS (Table 4.4). However, mice implanted with LPS pellets that consumed diets with added Se of 4 mg/kg had a significantly ($p<0.0001$) lower number of lymphocytes compared to all other groups. Number of neutrophils were significantly ($p<0.001$) higher

in mice fed diet with 0.2 mg Se/ kg and 4 mg Se /kg diets. No alteration in monocytes was observed by dietary Se or LPS.

Bone marrow T and B cells

Bone marrow Th-cells, T and B cells, lymphocytes, granulocytes and monocytes were analyzed using a flow cytometer and means with SEM are presented for each group of mice. Th-cells, B-cells and monocytes showed a significant increase with LPS as presented in Table 4.5 and 4.6, while granulocytes increased significantly with LPS compared to placebo in mice fed 2.0 mg Se/kg diet only. T-cells were significantly affected by dietary Se ($p<0.02$), and were significantly higher in mice fed the 4.0 mg Se/kg diet compared to other groups.

Cytokines and IgG2a Response

Neither Se intakes nor LPS stimulation significantly affected plasma IgG2a, IFNγ or IL-12p70 levels ($p>0.05$) (Table 4.7).

Discussion

An effective immune system is dependent on availability of key nutrients with antioxidant functions (Haddad 2002). Se is one of these nutrients and stimulates both innate and acquired immune responses due to its effect on cytokine production and regulation of ROS (Bhaskaram 2002; Rayman 2002). Se exerts its immune function through its regulatory action on redox balance (GSH-Px and TR) by effectively scavenging ROS (Thomson 2004; Sakr, Reinhart et al. 2007). Se also influences production of cytokines for effective immune response, (Regina 1999; Arthur, McKenzie

et al. 2003) and improves T-cell proliferation and immunity secondary to vaccine (McKenzie, S. Rafferty et al. 1998).

Se supplementation in humans showed increased proliferation of lymphocytes secondary to live polio vaccine virus and a greater clearance of the virus (Broome, McArdle et al. 2004; Pagmantidis, Méplan et al. 2008). The increase in lymphocyte numbers was associated with protective activity of GSH-Px and TR reducing free radicals that could destroy immune cells and preventing lipid peroxidases that lead to immune suppression secondary to metabolites of arachidonic acid (Daria, Cesare et al. 2008). The reported increases in leucocyte numbers with Se supplementation, however, were not consistent, as an experiment with aged mice showed only two thirds of mice had improved lymphocyte proliferation. This might be due to individual specific needs and difference in metabolic rate (Brown, Pickard et al. 2000)

In our study, we tested if dietary Se influenced immune responses to low grade inflammation induced by slow release LPS. Mice were fed varied concentrations of dietary Se for 90 days prior to stimulation by LPS for 28 days and tested for bone marrow immune cells, plasma cytokines and immunoglobulin levels. We used a torula-based Se depletion diet containing no added Se, as the basal diet similar to other experiments done in rodents (Knight and Sunde 1988; Cheng, Ho et al. 1997; Gomez, Solana et al. 2002). The deficient diet was well below daily requirements for rodents (0.1 mg/kg) (Subcommittee on Laboratory Animal Nutrition. National Research Council 1995). As the degree of immune response depends on the level of Se (Musik, Koziol-Montewka et al. 1999) and dose of inflammatory stimulants, in our study mice were fed with deficient Se, 0.2 mg Se/kg, 2.0 mg Se/kg and 4.0 mg Se/kg diets.

The results of our study were consistent with the notion (in humans) that Se must be taken above the physiologic level to enhance immunity (Kiremidjian-Schumacher, Roy et al. 1994). Our results showed a shift to an increased number of T-cells (CD3+) with the highest dietary Se, even though the total number of lymphocytes was not increased. This supports the preferential effect of Se in enhancing T-cells for immune response (Arthur, McKenzie et al. 2003). Although T-cells are not the only type of immune cells affected by Se level (Safir, Wendel et al. 2003; Kim, Johnson et al. 2004; Hoffmann 2007), they are important for further differentiation into Th-cells to enhance a variety of immune responses. The effect of low Se intake on T-cells indicates the importance of Se for T-cells proliferation for optimum immune response.

When plasma cytokines levels and IgG2a were analyzed, our results showed no difference by Se intake and LPS, despite the increase in bone marrow early blast cells, and monocyte numbers by LPS and T-cells by Se intake. In contrast to our finding, a study by Yusuke in dendritic cells which investigated the effect of low dose LPS (1ng/ml) on IL-12 production (at early stages of infection), showed increased IFNγ and induced IL-12 production (Saito, Yanagawa et al. 2006). This may be due to the effect of LPS on TNFα and IFN γ production at initial stage of inflammation leading to increased IL-12 production. The reason for the absence of a difference with LPS in the production of IL-12p -70, in our study, may be due to the difference in the timing of collecting blood samples. In our study, blood samples were collected four weeks after initiation of inflammation, when the acute inflammatory response had already leveled off.

In conclusion, Se supplementation increased plasma Se level and number of bone marrow T-cells, while LPS increased production of bone marrow Th-cells, B-cells and

monocytes. Interactions between Se and LPS affected numbers of peripheral lymphocytes and neutrophils. It may be interesting to investigate the effect of Se on other immune molecules that take part in polarization of Th-cells to gain knowledge on possible role of Se in disease conditions that skew the polarization balance to the pro-inflammatory side.

Table 4.1: Composition of Diets by Added Se (mg/kg diet)

Added Se	**Se 0.00 mg/kg**	**Se 0.2mg/kg**	**Se 2.0 mg/kg**	**Se 4.0 mg/kg**
Formula	**g/kg**			
Torula Yeast	340.0	340.0	340.0	340.0
L-Cystine	3.0	3.0	3.0	3.0
Dextrose, Monohydrate	399.02	399.02	399.02	399.02
Sucrose	100.0	100.0	100.0	100.0
Soybean Oil	60.0	60.0	60.0	60.0
Cellulose	50.0	50.0	50.0	50.0
Mineral Mix	35.0	35.0	35.0	35.0
Vitamin Mix AIN-93-VX	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5

Table 4.2: Body and Thymus Weight of Mice Fed Supplemental Se with or without LPS (Mean ±SE)

Added Se (mg/kg diet)	**Pellet**	**n**	**Body Weight (gm)**	**n**	**Thymus Weight (gm)**
0.0	Placebo	10	25.7±0.2	10	0.04± 0.00
0.0	LPS	8	24.1±0.5	8	0.05± 0.00
0.2	Placebo	11	26.0±1.2	11	0.04± 0.00
0.2	LPS	10	25.1±0.2	10	0.05± 0.00
2.0	Placebo	11	25.7±0.6	11	0.05± 0.00
2.0	LPS	10	26.1±0.7	10	0.05± 0.00
4.0	Placebo	10	24.8±0.3	10	0.04± 0.00
4.0	LPS	9	25.3±0.4	10	0.05± 0.00
Treatment Means					
Added Se					
0.0		18	25.0±0.3	18	0.05±0.00
0.2		21	25.5±0.3	21	0.05±0.00
2.0		21	25.9±0.4	21	0.05±0.00
4.0		19	25.0±0.3	20	0.05±0.00
LPS					
Placebo		42	25.6±0.2	42	0.043 ±0.00
LPS		37	25.0±0.3	38	0.050 ±0.00
P Values					
Se			0.13		0.86
LPS			0.06		0.06
Se * LPS			0.28		0.39

Table 4.3: Plasma Se of Mice Fed Supplemental Se with and without LPS (Mean±SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**Plasma Selenium (mg/L)**
0.0	Placebo	5	0.096 ± 0.030
0.0	LPS	5	0.103 ± 0.005
0.2	Placebo	5	0.222 ± 0.052
0.2	LPS	4	0.223 ± 0.050
2.0	Placebo	4	0.166 ± 0.060
2.0	LPS	5	0.250 ± 0.040
4.0	Placebo	5	0.188 ± 0.030
4.0	LPS	5	0.206 ± 0.031
Treatment Means			
Added Se			
0.0		10	0.099 ± 0.026[b]
0.2		9	0.222 ± 0.033[a]
2.0		9	0.212 ± 0.030[a]
4.0		10	0.197 ± 0.020[a]
LPS			
Placebo		19	0.168 ± 0.022
LPS		19	0.193 ± 0.023
P Values			
Se			<0.03
LPS			0.39
Se * LPS			0.79

Means with superscripts not sharing a common letter are significantly different

Table 4.4: Total White Blood Cells (WBC) and Differential Counts of Mice Fed Supplemental Se with or without LPS (Mean ± SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**Total WBC ($1\ 10^6$/mL)**	**Lymphocytes ($1\ x10^6$/mL)**	**Neutrophils ($1\ x10^6$/mL)**	**Monocytes ($1\ x10^6$/mL)**
0.0	Placebo	10	4.58 ± 0.47	$3.73±0.34^{a}$	$0.685 ±0.015^{bc}$	0.090± 0.020
0.0	LPS	8	4.38 ± 0.31	$3.54 ± 0.28^{a}$	$0.606 ± 0.061^{bc}$	0.149± 0.002
0.2	Placebo	11	3.72 ± 0.38	$3.25 ± 0.33^{a}$	$0.370 ± 0.053^{d}$	0.075± 0.015
0.2	LPS	10	4.01 ± 0.33	$3.14 ± 0.27^{a}$	$0.647 ± 0.056^{bc}$	0.098± 0.020
2.0	Placebo	11	4.40 ± 0.32	$3.77 ± 0.29^{a}$	$0.526 ± 0.034^{cd}$	0.086± 0.020
2.0	LPS	10	4.12 ± 0.32	$3.27 ± 0.26^{a}$	$0.638 ±0.064^{bc}$	0.010 ±0.002
4.0	Placebo	10	4.71 ± 0.27	$3.80± 0.21^{a}$	$0.760 ± 0.061^{b}$	0.093± 0.003
4.0	LPS	10	3.67 ± 0.25	$2.11± 0.15^{b}$	$1.480 ± 0.012^{a}$	0.042± 0.019
Treatment Means						
Added Se						
0.0		18	4.50±0.29	3.60±0.21	0.660±0.06	0.112±0.02
0.2		21	3.90±0.23	3.20±0.19	0.510±0.05	0.870±0.01
2.0		21	4.23±0.23	3.52±0.19	0.580±0.05	0.940±0.01
4.0		20	4.19±0.23	2.96±0.19	1.120±0.52	0.680±0.01
LPS						
Placebo		42	4.34±0.19	3.63 ±0.15	0.578±0.040	0.086±0.011
LPS		38	4.02±0.15	2.99 ±0.15	0.862±0.073	0.095±0.010
P Values						
Se			0.36	<0.068	<0.0001	0.13
LPS			0.20	<0.002	<0.0001	0.45
Se * LPS			0.27	<0.020	<0.0001	0.09

Means with superscripts not sharing a common letter are significantly different

Table 4.5: Number of T helper-cells, T-cells and B-cells in Bone Marrow of Mice Fed Supplemental Se with and without LPS (Mean ±SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**T- helper Cells ($1 x10^6$ /mL)**	**T- Cells ($1 x10^6$/mL)**	**B - Cells ($1 x10^6$ /mL)**
0.0	Placebo	6	0.54 ± 0.09	0.27 ± 0.04	3.40± 0.23
0.0	LPS	6	0.65 ± 0.08	0.31 ± 0.06	3.17 ± 0.52
0.2	Placebo	6	0.54 ± 0.09	0.31 ± 0.02	2.76 ± 0.45
0.2	LPS	6	0.64 ± 0.05	0.32 ± 0.06	3.98 ± 0.51
2.0	Placebo	5	0.29 ± 0.01	0.13 ± 0.05	1.86 ± 0.21
2.0	LPS	6	1.12 ± 0.28	0.60 ± 0.01	3.84 ± 0.80
4.0	Placebo	6	0.69 ± 0.01	0.52 ± 0.01	2.14 ± 0.30
4.0	LPS	6	1.26 ± 0.29	0.92 ± 0.02	2.38 ± 0.49
Treatment Means					
Added Se					
0.0		12	0.595±0.059	$0.289±0.036^b$	3.29±0.27
0.2		12	0.634±0.148	$0.313±0.071^b$	3.43±0.38
2.0		11	0.685±0.189	$0.366±0.089^b$	2.85±0.49
4.0		12	0.975±0.174	$0.722±0.144^a$	2.26±0.28
LPS					
Placebo		23	$0.534 ±0.091^b$	0.306 ±0.056	$2.53±0.12^b$
LPS		24	$0.906 ±0.112^a$	0.539 ± 0.082	$3.35±0.31^a$
P Values					
Se			0.20	<0.02	0.09
LPS			<0.01	<0.09	<0.02
Se * LPS			0.15	0.14	0.12

Means with superscripts not sharing a common letter are significantly different

Table 4.6: Numbers of Bone Marrow Early blasts, Lymphocytes, Granulocytes and Monocytes in Mice Fed Supplemental Se with or without LPS (Mean ± SE)

			Lymphocytes ($CD31^{mid}$ LY-6Cneg)	Granulocytes (CD31 neg LY-6C ^{med})	Monocytes (CD31neg LY-6C ^{hig})
Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**(1 x10^6/mL)**	**(1 x10^6/mL)**	**(1 x10^6/mL)**
0.0	Placebo	6	1.46 ± 0.39	0.296 ± 0.045^{b}	0.992 ± 0.127
0.0	LPS	6	1.35 ± 0.28	0.253 ± 0.051^{bc}	0.872 ± 0.141
0.2	Placebo	6	1.56 ± 0.25	0.257 ± 0.039^{bc}	0.833 ± 0.098
0.2	LPS	6	1.61 ± 0.30	0.355 ± 0.034^{ab}	1.210 ± 0.147
2.0	Placebo	5	0.71 ± 0.07	0.154 ± 0.025^{c}	0.553 ± 0.084
2.0	LPS	6	1.69 ± 0.29	0.452 ± 0.071^{a}	1.320 ± 0.189
4.0	Placebo	6	1.03 ± 0.21	0.224 ± 0.044^{bc}	0.683 ± 0.139
4.0	LPS	6	1.01 ± 0.20	0.324 ± 0.068^{ab}	1.250 ± 0.341
Treatment Means					
Added Se					
0.0		12	1.40±0.15	0.274±0.033	0.933±0.092
0.2		11	1.59±0.19	0.311±0.029	1.041±0.106
2.0		12	1.20±0.21	0.303±0.057	0.938±0.152
4.0		12	1.02±0.14	0.274±0.041	0.966±0.196
LPS					
Placebo		23	1.19±0.13	0.233±0.025	0.765 ± 0.090^{b}
LPS		24	1.42±0.11	0.346±0.025	1.160 ± 0.088^{a}
P Values					
Se			0.104	0.86	0.95
LPS			0.180	<0.003	<0.003
Se * LPS			0.079	<0.014	0.08

Means with superscripts not sharing a common letter are significantly different

Table 4.7: Plasma Levels of Pro-inflammatory Cytokines and IgG2a in Mice Fed Supplemental Se with and without LPS (Mean ±SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**IgG2a(ng/mL)**	**IFNγ (ng/mL)**	**IL-12(ng/mL)**
0.0	Placebo	5	7.93±2.23[b]	3.55±1.20	33.20±6.61
0.0	LPS	6	9.63±1.92[b]	2.95±0.86	29.50±3.96
0.2	Placebo	6	11.48±1.27[b]	1.52±0.16	25.84±2.75
0.2	LPS	6	7.98±1.12[bc]	2.50±0.36	33.50±8.20
2.0	Placebo	5	10.60±2.67[bc]	2.57±0.37	23.94±5.06
2.0	LPS	6	11.53±1.29[bc]	2.72±0.49	24.24±7.28
4.0	Placebo	6	5.12±1.46[c]	3.92±0.75	30.37±6.17
4.0	LPS	6	14.59±1.37[a]	1.81±0.30	26.89±5.00
Treatment Means					
Added Se					
0.0		11	8.85±1.41	3.19±0.67	31.35±3.72
0.2		12	9.73±0.96	1.94±0.25	29.67±4.28
2.0		11	11.06±1.42	2.96±0.29	24.10±4.37
4.0		12	9.85±1.72	2.87±0.50	28.63±3.82
LPS					
Placebo		23	8.82 ± 1.06	2.96±0.38	28.53±2.62
LPS		24	10.93 ± 0.85	2.99±0.30	28.53±3.04
P Values					
Se			0.63	0.41	0.66
LPS			0.08	0.40	0.96
Se * LPS			<0.01	0.14	0.74

Means with superscripts not sharing a common letter are significantly different

CHAPTER V

THE EFFECT OF DIETARY SELENIUM SUPPLEMENTATION AND LOW GRADE INFLAMMATION ON PLASMA GLUTHATHIONE PEROXIDASE, SELENIUM AND TOTAL ANTIOXIDANT CAPACITY IN C57BL/6 MICE

Abstract

Selenium is an important component of glutathione peroxidase (GSH-Px) enzyme. The level of GSH-Px activity is dependent on adequacy of Se in the host. We examined the level of plasma GSH-Px activity in mice fed different concentrations of dietary selenium (Se) and challenged with Lipopolysaccharide (LPS) in an experimental study. Plasma GSH-Px and Total Antioxidant Capacity (TAC) were assessed using commercially available kits while ICP-MS was used to measure plasma Se. The effect of graded dietary Se intake in mice on body composition and biochemical markers were also investigated. Liver function tests were also performed using a clinical analyzer. GSH-Px and TAC were significantly increased by dietary Se.

GSH-Px activity increased in mice from 34.1 mU/L in the Se 0.0 mg/kg diet group to 1024.8 mU/L in the Se 4mg/kg diet group ($p<0.0001$). Mice in the 0.2 mg/kg and 2.0mg/kg diet groups showed GSH-Px activity of 851.5 mU/L and 909.1mU/L respectively. The TAC changed from 6.18 mM in mice fed 0 mg/kg Se added to diet to 7.62mM in mice fed 4.0mg/kg Se added to diet ($p<0.001$). Significant changes in plasma Se were observed with dietary Se level ($p<0.03$). Moreover, a significant increase

($p<0.036$) in plasma alkaline phosphatase (ALP) activity by dietary Se was observed. Future studies are recommended to demonstrate the interaction of Se with other antioxidant minerals such as copper and zinc to determine the minimum dose of Se required to increase the activity of GSH-Px enzymes that enhance immune response in chronic immunosuppressive conditions.

Introduction

The trace mineral Se is an essential nutrient in life. Se as selenocysteineis is a major part of selenoproteins (Sunde 1997). Some selenoproteins have enzymatic properties and play a role in the cellular reduction-oxidation (redox) system (Sunde 1997). For example, thioredoxinreductase (TR), which is one of the selenoenzymes, helps to reduce nucleotides in DNA synthesis and helps to regulate the intracellular redox state (Allan, Lacourciere et al. 1999). Selenium dependent gluthathione peroxidases are important for the reduction of hydrogen peroxide and hydroperoxidases into harmless byproducts such as water and alcohol (Diplock 1994; Sunde 1997). This redox function helps to maintain membrane integrity and further limits oxidation of lipids, lipoproteins and DNA leading to cardiovascular diseases and other chronic diseases.

The effects of Se supplementation seem to depend on the baseline level of Se in the host. The lower the baseline level the better is the response, as demonstrated in a study from Australia in patients with autoimmune thyroiditis (Moncayo, Moncayo et al. 2005). Although the range for normal level of plasma Se is wide, 40-140 µg/L, maximal activities of GSH-Px are exerted at Se concentration of 100 - 114 µg/L (Thomson, McLachlan et al. 2005). On the contrary, if the level of Se is above 200 µg/L, it may induce pro-oxidant adverse effects. Se, through its activated GSH-Px enzymes,

neutralizes effects of oxidative stress induced by various inflammatory agents (Ryan-Harshman and Aldoori 2005). Studies in *vitro* indicated that Se influences inflammation as a result of viral, bacterial or stress stimulation (Maehira, Luyo et al. 2002). For example, in severe inflammatory response, the production of reactive oxygen species increases and leads to severe tissue damage (Galley, Davies et al. 1996). In such situations, if the host's plasma Se is low, more damage to tissues and organs is expected (Sakr, Reinhart et al. 2007). On the contrary, Se supplementation, in severe inflammatory situations, resulted in reduced tissue damage and better clinical outcomes in an observational study (Angstwurm, Schottdorf et al. 1999). A multicenter, randomized controlled trial study also confirmed this finding that Se supplementation decreased mortality in patients with severe sepsis (Zamamiri-Davis, Lu et al. 2002).

Such anti-inflammatory effects of Se are also attributed to its immune boosting effect. Se supplementation significantly decreased the bacterial endotoxin lipopolysaccharide (LPS) induced tumor necrosis factor alpha (TNFα) and cyclooxygenase 2 (COX-2), by inhibiting MAP kinase pathways (Vunta, Belda et al. 2008). On the other hand, when Se level is low, high levels of TNFα increased activation of NFκB (Vunta, Belda et al. 2008). TNFα induces production of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin), necessary for pro-inflammatory response by recruiting immune cells (Zhang, Yu et al. 2002). In an *vitro* study using human umbilical vein endothelial cells, Se supplementation (as sodium selenite), in a dose dependent manner, also inhibited TNFα stimulated expression of adhesion molecules (Zhang, Yu et al. 2002). This study suggested that higher level of Se

may inhibit NF-κB via GSH-Px and attenuate inflammation. When the level of GSH-Px is high, it decreases the levels of ROS and IκB-α phosphorylation and consequently the translocation of NF-κB to the nucleus (Lun, Zhang et al. 2006). GSH-Px also conserves degradation of IκB-α and prolongs its half-life which keeps NF- κB in its inactive form (Kretz-Remy and Arrigo 2001). Based on this current knowledge, the present study investigated plasma Se, GSH-Px, and Total Antioxidant Capacity (TAC) in mice fed graded dietary Se and challenged with LPS.

Moreover, as the liver is the major detoxifying organ, our study also examined key proteins that illustrate liver functions in mice under low grade inflammatory stress induced by low levels of LPS. For this study we selected, aminotransaminase (ALT), alkaline phosphatase (ALP) and albumin (ALB) levels, as these proteins are related in the LPS detoxification pathways.

ALT, a marker of liver function, is synthesized mainly by liver and catalyzes transamination reaction between alanine and ketoglutarate leading to the formation of pyruvate and glutamate (Welch 1972; Alexis and Papaparaskeva-Papoutsoglou 1986). This role, makes ALT an important enzyme in glucose metabolism (Philip 1973). ALT is also used in clinical diagnosis of liver function in humans and is known to rise during viral infections, other liver diseases, toxicity and in diseases related to muscle and celiac disease (Chen, Huang et al. 2007). Recent findings indicated that ALT is up-regulated by LPS (Lun, Zhang et al. 2006) and for this reason we have evaluated level of ALT in mice.

ALB, is a protein synthesized by liver and its production is affected in cases of liver diseases. Studies have shown that physiologic levels of circulating ALB facilitates the interaction of LPS with LPS binding protein (LBP) and CD14 for recognition of

pathogens by TLR- 4 for immune response (Gioannini, Zhang et al. 2002). An *in vitro* experiment, with treatment of exogenous graded ALB, showed an increase in pro-inflammatory gene expression through activation of NF-κB (Drumm, Gassner et al. 2001; Drumm, Bauer et al. 2002).

Alkaline phosphatase (ALP) is present naturally in different organisms and mammalians (Millan and 2006). Most of the ALP enzymes are homodimers and contain three metal ions (two zinc and one magnesium) at each catalytic site. ALP plays a catalytic role in hydrolyzing monoesters of phosphoric acid and transphosphorylation reactions. ALP can also dephosphorylate various phosphorylated substrates including endotoxins such as LPS (Poelstra, Bakker et al. 1997). LPS, as a product of gram negative bacteria, contains two phosphate groups and ALP has been found to attenuate LPS induced inflammation in rats (Poelstra, Bakker et al. 1997). Thus, our study investigated plasma Se, GSH-Px, total antioxidant capacity (TAC), ALB, ALP and ALT in mice fed graded dietary Se and challenged with LPS.

Methods

Mice and diets

Forty-one timed-pregnant C57BL/6 mice (Harlan, Indianapolis, IN) were fed Se-deficient (modified AIN-93G) diets for the final 5-6 days of gestation. The animals were housed in an environmentally controlled animal care facility and delivered their litters approximately 5-6 days after arrival. The dams continued to receive the pre delivery Se-depleted diet for the three weeks of lactation. At 24 days pups were weaned and randomly assigned to one of the four dietary groups (Table 5.1) which they were fed until

120 days of age. Diet was provided daily and water bottles and beddings on a weekly basis. Mice were fed *ad libitum* (approximately 5 g diet/day/mice). At 90 days of dietary treatment, mice within each diet group were assigned to 0 or 0.1µg/g/d of time-release LPS (E.coli Serotype 0127:B8) pellet to produce a very low grade inflammatory stress. Treatment was maintained (time-release LPS or placebo pellet) for 28 days and the mice continued to be fed their respective diets throughout the study.

Plasma GSH-Px

Plasma GSH-Px was measured using a kinetic enzyme assay as per the protocol provided by the manufacturer (Oxford Biomedical Research, Inc. Oxford, MI). The assay was carried out at room temperature and the spectrophotometer was set at 340 nm. The spectrophotometer was zeroed at 340 nm using deionized water. Appropriate volume of assay buffer, pre diluted NADH reagent and the sample were pipetted into a cuvette and placed in the spectrophotometer followed by addition and mixing of tert-butyl hydroperoxide by pipetting. GSH-Px enzyme activity was measured at A340 for three minutes.

Total Antioxidant Capacity (TAC)

Antioxidant assay kit was used to measure TAC in plasma samples (Cayman Chemical Company, Ann Arbor, MI). The assay procedure was done in accordance with the manufacturer's protocol. The assay measured the ability of antioxidants in the samples of plasma in inhibiting oxidation of 2, 2´-azino-di (3-ethylbenzothiazoline sulphonate) ABTS to ABTS•+ by Metmyoglobin. The TAC of samples preventing oxidation of ABTS was compared to the Trolox standard (water soluble tocopherol analog) and quantified as millimolar Trolox equivalents.

Plasma Se

Plasma Se was analyzed by Inductively Coupled Plasma Mass Spectrometer (ICPMS) (Elan 9000, Perkin Elmer, and Norwalk, CT). All plasma samples were diluted 50 fold (40 µl diluted to 1.96 ml) with 0.1% HNO3 (GFS Chemicals, Powell, OH) in deionized water (Milli-Q, Advantage A10, Millipore, France). Standard solutions of Se were prepared by dilution of certified standard solutions (Perkin Elmer, Norwalk, CT). Diluted working standards were prepared immediately prior to their use by diluting an intermediate stock standard solution. All samples and standards were spiked with 4 µg/L gallium as an internal standard. Quality control samples (Utak Laboratories, Inc., Valencia, CA) were utilized in order to verify method performance was within the recommended ranges.

Clinical Analyzer

A Biolis 24i clinical chemistry analyzer was used to determine plasma level of ALT, ALP and ALB. The necessary kits were purchased from Carolina Liquid Chemistries Corp. and manufacturer's instructions were strictly followed.

Statistical Analysis

All statistical tests for comparison of means were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA). GLM procedure tested the main effects of diet and LPS on GSH-Px, TAC, ALT, ALP and ALB. A least square means post hoc test was used to identify the means that differed. Differences were considered significant at $p < 0.05$.

Results

GSH-Px Activity

When mice in different dietary Se groups were compared, the group that consumed no added dietary Se showed significantly lower GSH-Px activity than the other three dietary Se groups containing 0.2, 2.0 and 4.0 mg Se /kg of diet ($p<0.0001$, see Table:5.2). The diet with 0.2 mg Se added/kg represented the control diet.

Plasma Se

Plasma Se of mice from different experimental diets at necropsy showed significant differences by dietary Se concentration ($p<0.03$). Mice on no added dietary had significantly lower plasma Se level compared to other groups (Table 5.3). However, plasma Se was not significant by LPS.

Plasma TAC

Results of plasma TAC in mM/L are presented in Table 5.4 below. Although the TAC values are found in a narrow range (5.46 – 8.85 mM/L), statistically significant difference ($p<0.0001$) was observed by dietary Se. The slight decrease in TAC values for mice in the Se deficient group indicates the role of dietary Se in enhancing plasma TAC values in the remaining dietary Se groups.

Plasma ALT, ALP and ALB

There was a significant difference ($p<0.03$) in plasma ALP by dietary Se, after 120 days of dietary treatment (Table 5.5). The mice receiving 0.02 mg/kg and 2.0 mg/kg had significantly higher levels of ALP activity compared to other groups. No differences were observed in plasma ALT and ALB based on either Se or LPS.

Discussion

Se is considered an essential element at the level of 0.1 mg/kg diet for animals (Clement 1998; Chen and Berry 2003). Se provides additional benefits (cancer prevention

and immune boosting) in animals at the range of 1-5 mg/kg diet (Clement 1998). In our experiment, we fed mice diets with added Se concentration (0.2, 2.0 and 4.0 mg/kg diet). This was done taking into consideration the enhanced effect of Se supplementation on Se dependent antioxidant enzymes and TAC.

In our study, we found that GSH-Px, TAC and plasma Se in mice increased with dietary Se intake. Mice that consumed Se deficient diet in our study (0.0mg/kg) showed significantly lower levels of these biomarkers ($p<0.05$), which is in line with earlier work on the effect of long term Se deficiency in rats (Wu, Huang et al. 2003). In their study the Se deficient rats showed significantly lower TAC, but with an increase after one month of Se supplementation (Wu, Huang et al. 2003). Another study also showed an increase in TAC of rat heart muscle with Se supplementation (Danesi, Malaguti et al. 2006). The TAC assay in our study measured the ability of antioxidants in the samples of plasma in inhibiting oxidation of ABTS to ABTS•+ by Metmyoglobin. Plasma Se correlated with GSH-Px ($r = 0.32$; $p<0.05$) and the GSH-Px correlated with TAC level as well ($r = 0.55$; $p<0.0005$, data not shown). This signifies the role of plasma Se in increasing both GSH-Px and TAC levels in the plasma. TAC reflects the collective contribution of both enzymatic and non-enzymatic antioxidant molecules. It includes the contribution of each antioxidant molecule's capacity of reducing the potency of free radicals. A significant effect of Se on plasma ALP was also observed in our study. The activity of ALP was significantly lower in the 0.2 and 4.0 groups than in the deficiency and 2.0 mg Se/kg diet groups. The reason for the differences in ALP was unclear.

Table 5.1: Composition of Diets by Added Se (mg/kg diet)

Added Se	**Se 0.00 mg/kg**	**Se 0.2mg/kg**	**Se 2.0 mg/kg**	**Se 4.0 mg/kg**
Formula	**g/Kg**			
Torula Yeast	340.0	340.0	340.0	340.0
L-Cystine	3.0	3.0	3.0	3.0
Dextrose, Monohydrate	399.02	399.02	399.02	399.02
Sucrose	100.0	100.0	100.0	100.0
Soybean Oil	60.0	60.0	60.0	60.0
Cellulose	50.0	50.0	50.0	50.0
Mineral Mix	35.0	35.0	35.0	35.0
Vitamin Mix AIN-93-VX	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5

Table 5.2: Plasma GSH-Px Activity in Mice Fed Supplemental Se with and without LPS (Mean ± SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**Plasma GSH-Px mU/mL**
0.0	Placebo	5	32.5 ± 11.6
0.0	LPS	5	36.6 ± 5.4
0.2	Placebo	5	937.5 ± 148.4
0.2	LPS	5	725.4 ± 139.3
2.0	Placebo	5	977. 4 ± 100.5
2.0	LPS	5	880.8 ± 144.1
4.0	Placebo	5	1039.2 ± 161.9
4.0	LPS	5	1010.4 ± 165.9
Treatment Means			
Added Se			
0.0		10	34.1 ± 89.2[b]
0.2		10	851.5 ± 89.1[a]
2.0		10	909.1 ± 89.1[a]
4.0		10	1024.8 ± 89.1[a]
LPS			
Placebo		20	765.24±106.2
LPS		20	638.10±107.6
P Values			
Se			<0.0001
LPS			0.36
Se * LPS			0.74

Means with superscripts not sharing a common letter are significantly different

Table 5.3: Plasma Selenium Level in Mice Fed Supplemental Se with and without LPS

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**Plasma Selenium (mg/L)**
0.0	Placebo	5	0.096 ± 0.030
0.0	LPS	5	0.103 ± 0.005
0.2	Placebo	5	0.222 ± 0.052
0.2	LPS	4	0.223 ± 0.050
2.0	Placebo	4	0.166 ± 0.060
2.0	LPS	5	0.250 ± 0.040
4.0	Placebo	5	0.188 ± 0.030
4.0	LPS	5	0.206 ± 0.031
Treatment Means			
Added Se			
0.0		10	0.099 ± 0.026[b]
0.2		9	0.222 ± 0.033[a]
2.0		9	0.212 ± 0.030[a]
4.0		10	0.197 ± 0.020[a]
LPS			
Placebo		19	0.168 ± 0.022
LPS		19	0.193 ± 0.023
P Values			
Se			<0.03
LPS			0.39
Se * LPS			0.79

Means with superscripts not sharing a common letter are significantly different

Table 5.4: Plasma Total Antioxidant Capacity in Mice Fed Supplemental Se with and without LPS (Mean ±SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**Plasma TAC (mM/L)**
0.02	Placebo	5	6.58± 0.46
0.02	LPS	5	5.46± 0.93
0.2	Placebo	5	7.91± 0.52
0.2	LPS	5	8.02± 0.32
2.0	Placebo	5	8.39 ±0.69
2.0	LPS	5	8.85 ±0.39
4.0	Placebo	5	8.48 ±0.38
4.0	LPS	5	8.28 ±0.38
Treatment Means			
Added Se			
0.02		10	6.18 ± 0.36^{b}
0.2		10	7.27 ± 0.36^{a}
2.0		10	7.46 ± 0.36^{a}
4.0		10	7.62 ± 0.36^{a}
LPS			
Placebo		20	7.29±0.22
LPS		20	7.10±0.32
P Values			
Se			<0.001
LPS			0.62
Se * LPS			0.49

Means with superscripts not sharing a common letter are significantly different

Table 5.5: Plasma Albumin, Alkaline phosphatase and Alanine transaminase in Mice Fed Supplemental Se with and without LPS (Mean ± SE)

Added Dietary Se (mg/kg diet)	**Pellet**	**n**	**ALB mg/dL**	**ALP mg/dL**	**ALT mg/dL**
0.0	Placebo	6	2.59 ± 0.11	4.5 ± 0.99	11.8 ± 1.05
0.0	LPS	6	2.53 ± 0.84	4.3 ± 1.54	11.7± 0.95
0.2	Placebo	6	2.55 ± 0.08	2.5 ± 1.02	9.5 0 ± 0.99
0.2	LPS	6	2.65 ± 0.11	2.5 ± 0.62	11.6 ± 1.50
2.0	Placebo	6	2.57 ± 0.11	4.2 ± 1.08	11.3 ± 0.56
2.0	LPS	6	2.27 ± 0.32	5.2 ± 0.70	13.3 ± 2.81
4.0	Placebo	6	2.63 ± 0.06	1.7 ± 0.61	11.2 ± 2.88
4.0	LPS	6	2.48 ± 0.07	3.2 ± 0.79	13.2 ± 1.19
Treatment Means					
Added Se					
0.0		12	2.56±0.65	4.4±0.68[a]	11.8±1.19
0.2		12	2.60±0.57	2.5±0.68[b]	10.6±1.26
2.0		12	2.57±0.06	4.7±0.68[a]	12.3±1.19
4.0		12	2.54±0.04	2.4±0.68[b]	12.2±1.19
LPS					
Placebo		24	2.58±0.04	3.21±0.50	10.95±0.78
LPS		23	2.48±0.88	3.79±0.50	12.48±0.85
P Values					
Se			0.95	<0.036	0.73
LPS			0.77	0.40	0.22
Se * LPS			0.61	0.50	0.88

Means with superscripts not sharing a common letter are significantly different

CHAPTER VI

SUMMARY AND CONCLUSIONS

Summary

Se, as an important element for optimum immune function, works by promoting both the innate and acquired immune systems (Brown and Arthur 2001). The selenoproteins are very important in regulating antioxidant and redox systems thereby influencing membrane integrity and thus guarding against DNA damage (Arthur 2003). The antioxidant effect of Se is mediated through GSH-Px, which plays an antioxidant role by removing free radicals produced during normal metabolism and oxidative stress. Se deficiency leads to decreased levels of GSH-Px and reduced ability of producing respiratory burst reaction by neutrophils and macrophages, which is important in killing the microbes (Arthur 2003). The role of Se in up regulating IL-2 receptors for effective cellular and humoral immune responses is well documented (Arthur 2003). Overall, the existing body of literature consistently indicated that Se has a role in maintaining the immune system. However, this evidence does not indicate the actual threshold of Se concentration required for optimum immune system function beyond its classical antioxidant functions.

In our study, dams were fed a Torula-yeast based Se depletion diet for the final week of gestation and through lactation. At 23 days, pups were weaned to the depletion

diet or to diets with 0.2, 2 or 4 mg/kg added Se. Se was added as sodium selenate for 14 weeks. Four weeks before necropsy, lipopolysaccharide (LPS) time-release pellets (0 or 0.1μg/g body weight/d) pellets were implanted subcutaneously. At necropsy the bone marrow from femur was flushed with saline, labeled with fluorochrome conjugated primary antibodies (CD3, B220, CD4, CD31 and LY-C6) and analyzed by flow cytometry (FACS). LPS introduced a significant ($p<0.05$) increase in number of T- helper cells (CD4+) ($0.534x10^6$/mL vs $0.906x10^6$/mL, B-cells (B220+) ($2.53x10^6$/mL vs $3.35x10^9$/L), and monocytes (CD31neg LY-$C6^{hig}$) ($0.765\ x10^6$/mL vs $0.088\ x\ 10^6$/mL) compared to placebo groups. In addition T-cell numbers were greater with increasing Se intake ($p<0.02$).

Plasma GSH-Px and TAC were significantly increased by Se intake ($p<0.001$). GSH-Px activity increased in mice from 34.1 mU/L in the Se depletion group to 1024.8 mU/L in the group fed with 4 mg/kg added dietary Se ($p < 0.0001$). Mice in the 0.2 mg/kg and 2.0mg/kg diets showed GSH-Px activity of 851.5 mU/L and 909.1 mU/L respectively. The TAC was also shown to change from 6.18 mM in mice fed the depletion diet to7.62 mM in mice fed diet with 4.0 mg Se added /kg diet ($p< 0.001$). Statistically significant change was also observed in plasma Se by dietary Se concentration ($p< 0.03$).

In sum, plasma Se and GSH-Px activities increased with added dietary Se. The GSH-Px activity plateaued after 0.2 mg added Se/kg diet and looked unlikely to increase further with any increase in the concentration of dietary Se. The increased GSH-Px activity by dietary Se was expected to down-regulate inflammation. However, the differential WBC count increased for mice fed 2 mg/kg and 4 mg kg diets, even though

the total white blood cells showed no significant difference among the groups. This could be as a result of the additional inflammatory effect of very high Se intake. The bone marrow immune cells such as monocytes, Th-cell and B-cell also increased in numbers in response to the low grade inflammatory model used in our study despite the difference in Se intake. The difference in monocyte count between peripheral and bone marrow was unclear but may be that all cells produced in bone marrow may not be released peripherally as expected. Our study also observed null difference in the inflammatory markers assessed due to either Se or LPS. We found significant negative correlation between TAC and level of IL-12 and IFNγ in the plasma ($r = -0.36$ and $r = -0.31$) respectively. The relatively higher level of TAC in all groups compared to Se deficient group might have contributed to down- regulating the production of these cytokines in addition to the low grade of inflammation used in our study. Overall, Se selectively affected T-cells even in this low grade inflammation model. The observed difference in the subset of leukocytes in bone marrow cells by LPS was expected although the difference may not be as high as with a high grade inflammation model with increased production of cytokines.

Conclusion

The hypothesis that Se can affect the immune system is supported by a consistent body of scientific evidence. Surprisingly though, limited knowledge exists on the use of Se as an immune enhancing therapy clinically. The possibility that Se supplementation can boost immunity warrants further well-planned clinical trials.

Research Needs

Well-designed experimental research in animal models is necessary to establish the active metabolite of Se that is important for immune response. Some of the suggested areas of research are mentioned below.

- ***Experimental Studies:*** Randomized experimental studies using defined Se-compounds with specific endpoints in the immune system are warranted. This may generate evidence for the type of Se compound which is more effective in boosting the immune system.
- ***Chemical forms of Se Compounds:*** Methods are needed to characterize the chemical forms of Se present in biological tissues. These would help to formulate the appropriate chemical form of Se for supplementation for clinical trials to enhance the immune system.
- ***Doses of Se:*** A better understanding is required to identify the doses of different chemical forms of Se for effective immune enhancement. The studies should also determine the minimal effective dose and assess safety.

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APPPENDICES

B. Oklahoma State University Institutional Animal Care and Use Approval Form

VITA

HANA NEKATEBEB BEKELE

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECT OF SDIETARY SELENIUM INTAKE AND LIPOPOLYSACCHARIDE STIMULATION ON SELECTED IMMUNE AND INFLAMMATORY MARKERS IN C57BL/6 MICE

Major Field: Human Sciences (Option: Nutritional Sciences)

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Human Sciences (Nutritional Sciences) at Oklahoma State University, Stillwater, Oklahoma in May, 2012.

Completed the requirements for the Master of Science in Community Health at University of Heidelberg, Heidelberg/Germany in 1995.

Completed the requirements for the Bachelor of Science in Medicine at Addis Ababa University, Addis Ababa, Ethiopia in 1983.

Experience:
Worked as Medical Nutrition Researcher at the Ethiopian Nutrition Institute from 1985 to 1996. Worked as Child Survival Specialist at USAID/Ethiopia from 1997 to 2001. Served as Deputy Regional Coordinator for LINKAGES/Ethiopia project from 2003 to 2006 and as Regional Child Health Team Leader for Regional Center Quality of Health Care project funded by USAID/East Africa in 2007. Worked as Senior Nutrition Consultant in 2008 for FANTA/AED project.

Professional Memberships:
Food and Nutrition Society of Ethiopia (FONSE), Ethiopian Public Health Association (EPHA) and Ethiopian Student Association at Oklahoma State University (ESA-OSU).

Name: Hana Nekatebeb Bekele Date of Degree: May, 2012

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: THE EFFECTS OF DIETARY SELENIUM INTAKE AND LIPOPOLYSACCHARIDE STIMULATION ON SELECTED IMMUNE AND INFLAMMATORY MARKERS IN C57BL/6 MICE

Pages in Study: 109 Candidate for the Degree of Doctor of Philosophy
Major Field: Human Sciences (Nutritional Sciences)

Scope and Method of Study:
This was an experimental study which investigated the effect of dietary selenium and LPS stimulation on immune markers of T and B cells in C57BL/6 mice by using bone marrow cell numbers, peripheral white blood cells counts and biochemical markers.. Flowcytometer analysis was done to assess number of B and T cells, lymphocytes, granulocytes and monocytes in bone marrow. Liver functions (ALP, ALT and ALB) were assessed using a clinical analyzer. Commercially procured enzyme linked immunsorbant assay (ELISA) kits were used to analyze IL-12, IFNγ and IgG2a; plasma Se was analyzed using an inductively coupled plasma mass spectrometer (ICP-MS). Plasma glutathione peroxidase (GSH-Px) activity was analyzed using a kinetic assay and the total antioxidant capacity (TAC) was analyzed using a colorimetric method.
Findings and Conclusions: An amount of inflammation that did not significantly reduce body weight and showed no clinical signs of illness caused differences in most of the biomarkers selected for this study. At necropsy, plasma Se was significantly affected by dietary Se intake (0.099±0.026 mg/L in Se deficient group to 0.197±0.020 mg/L for 4mgSe/kg group). Interaction affected peripheral differential counts of white blood cells. Mice fed 4 mg Se added/kg diet with LPS had significantly lower numbers of lymphocytes compared to other groups. Peripheral neutrophil numbers were significantly higher for mice fed 4 mg Se added/kg diet with LPS and lower for mice fed 0.2 mg Se added/kg diet compared with placebo. LPS introduced a significant ($p<0.05$) increase in number of Th-cells (CD4+) ($0.534x10^6$/mL vs $0.906x10^6$/mL, B-cells (B220+) ($2.53x10^6$/mL vs $3.35x10^9$/L), and monocytes (CD31neg LY-$C6^{hig}$) ($0.765\ x10^6$/mL vs $0.088\ x\ 10^6$/mL) compared to placebo groups. In addition T-cell numbers were greater with 4.0 mg Se /kg diet ($p<0.02$). GSH-Px activity increased in mice from 34.057 mU/L in Se 0.02 mg/kg diet group to 1024.82 mU/L in Se 4mg/kg diet group ($p<0.0001$).The TAC was shown to change from 6.02 mM in mice fed deficient diet to 8.38 mM in mice fed with 4.0 mg/kg added Se diet ($p<0.001$). Significant changes in plasma alkaline phosphatase ($p<0.036$) level by dietary Se were also observed. The hypothesis that Se can affect the immune system is supported by a consistent body of scientific evidence including the results of our study. An evaluation of the possible therapeutic use of Se supplementation to enhance immunity warrants further well-planned experimental studies.

ADVISER'S APPROVAL: Dr. Barbara J Stoecker