The Effects of Selenomethionine and Wheat Biofortified with Selenium on DNA Damage and Cell Death in Human Lymphocytes

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Abstract

Selenium (Se) is an essential micronutrient, being a component of more than twenty seleno-proteins in humans. Previous studies suggested that increased intake of Se may reduce the risk of degenerative diseases including cancer; however, excessive intake can be toxic. Wheat is one of the major dietary sources of Se in humans, mainly in the form of L-selenomethionine (Se-met) but the impact of this source of Se on human health at the genome level was previously unexplored.

This PhD project aimed to (a) determine the safe dose-range and bio-efficacy of Se-met *in vitro*; (b) identify the optimal concentration of Se-met for reduction of genome damage *in vitro*; (c) investigate the optimal concentration of Se-met for improving resistance to gamma radiation or hydrogen peroxide induced genome damage *in vitro*; d) determine the bioavailability and bioefficacy of Se *in vivo*, in the form of either Se-met or wheat biofortified with Se; e) identify the nutrients and food groups that are correlated with Se intake/status and f) identify the nutrients, food groups and plasma mineral concentrations that are correlated to baseline lymphocyte DNA damage.

The *in vitro* study was performed on the peripheral blood lymphocytes isolated from six males and cultured with media supplemented with Se-met in a series of Se concentrations from 3 to 3850 µg Se/l while keeping the total methionine (i.e. Se-met + L-methionine) concentration constant. Baseline genome stability of lymphocytes and the extent of DNA damage induced by 1.5 Gy γ-ray or 7.5 µM hydrogen peroxide (H_2O_2) were investigated using the Cytokinesis-block Micronucleus Cytome (CBMN-Cyt) assay and the alkaline Comet assay with and without glycosylase (Fpg or Endo III) treatment after 9 days of culture. Results showed that high Se concentrations (≥1880 µg Se/l) caused strong inhibition of cell division, extensive DNA damage and increased cell death indicating cytotoxicity and genotoxicity. Baseline frequency of nucleoplasmic bridges (NPBs) and nuclear buds (NBud) declined significantly as Se concentration increased from 3 µg Se/l to 430 µg Se/l (P trend = 0.03 and 0.008, respectively); however, a significant trend of increase in Comet DNA damage was also observed (P trend <0.05) in lymphocytes. Selenium concentration (≤ 430 µg Se/l) had no significant effect on baseline frequency of micronuclei (MN) or DNA oxidation and had no protective effect against γ-ray-induced or H_2O_2-induced genome damage in lymphocytes.

A randomised double-blind placebo-controlled intervention trial was conducted on healthy South Australian males (n = 62, age (mean ± SD) 56 ± 7.0 years) with Se

dosage increased every 8 weeks for a total duration of 24 weeks. This study compared the bioavailability, by using plasma Se concentration as the biomarker, and bioefficacy of Se, by using platelet glutathione peroxidase (GPx) activity and lymphocyte DNA damage as biomarkers, from wheat process-fortified with Se-met (PROFORT) and high-Se wheat biofortified with Se (BIOFORT) compared to non-fortified normal (CONTROL) wheat. It was found that increased Se intake from BIOFORT wheat increased plasma Se concentration effectively in a dose-response manner from a baseline of 122 µg/l up to 190 µg/l ($P<0.001$). Increased Se intake from PROFORT wheat also increased plasma Se with a plateau at 140 µg/l, being therefore less effective than BIOFORT wheat ($P<0.001$). There was no significant change in Se status in the CONTROL group. Improved plasma Se concentrations had no effect on platelet GPx activity or lymphocyte DNA damage in either of the intervention groups.

Results from the food frequency questionnaire (FFQ) survey (n = 173) and plasma Se concentration survey (n = 179) suggested that the study population screened for participation in the *in vivo* trial described above had a mean plasma Se concentration (± SD) of 102 (± 12) µg/l and a mean (± SD) estimated Se intake of 165 (± 68) µg/d. This is a higher estimated Se intake than found in previous Australian studies. The major dietary sources of Se were found to be bread/cereals, fish/seafood and meat. However, increased intake of nuts/seeds, which are rich in Se, may have undesirable effects on lymphocyte DNA oxidation in this Se-replete population.

In conclusion, the *in vitro* studies suggest that (1) Se-met at higher concentrations at greater or equal to 1880 µg Se/l is cytotoxic; (2) Se-met may improve specific genome stability biomarkers such as nucleoplasmic bridge and nuclear bud at concentrations up to 430 µg Se/l, but further studies are needed to verify this effect. The *in vivo* studies in older men showed that Se from BIOFORT wheat is more effective in raising plasma Se concentration than Se from wheat process-fortified by the addition of Se-met, when both wheat products were subjected to strong heat. However, the platelet GPx activity and lymphocyte DNA damage appeared not to be modified by improved Se status.

This work contains two publications:

1) "The effect of selenium, as selenomethionine, on genome stability and cytotoxicity in human lymphocytes as measured by the cytokinesis-block micronucleus cytome assay". ***Mutagenesis*** 2009 May;24(3):225-32. 2) "Increased consumption of wheat biofortified with selenium does not modify biomarkers of cancer risk, oxidative stress or immune function in Australian males" ***Environmental Molecular Mutagenesis.*** 2009 July; 50 (6):489-501

The latter one was not able to be published in a journal of higher impact factor due to part of the data had been published elsewhere. Both articles are attached in Appendix.

Declaration

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"Increased consumption of wheat biofortified with selenium does not modify biomarkers of cancer risk, oxidative stress or immune function in Australian males" Epub ahead of print in ***Environmental Molecular Mutagenesis.*** DOI: 10.1002/em

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List of abbreviations

ACCV	Anti-Cancer Council of Victoria
AIDS	Acquired immunodeficiency syndrome
ALS	Alkali labile site
ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutated gene
ATP	Adenosine tri-phosphate
ATR	Ataxia telangiectasia mutated and Rad3-related gene
AU	Arbitrary unit
BIOFORT	Wheat biofortified with selenium
BNed	Binucleated
BRCA	Breast cancer gene
Ca	Calcium
CBMN Cyt assay	Cytokenesis-block micronucleus cytome assay
CHK2	Background checkpoint kinase 2 gene
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cu	Copper
$CuSO_4$	Copper sulphate
CV	Coefficient of variation
Cyto-B	Cytochalasin B
DAN	Diaminonaphthalene
DI	Deiodinase
DMABP	3,2'-dimethyl-4-aminobiphenyl
DMSO	Dimethyl sulfoxide
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
Endo III	Endoneclease III
FBS	Foetal bovine serum
Fe	Iron
FFQ	Food frequency questionnaire
Fpg	Formanidopyrimidine-DNA glycosylase

*Gadd*45	Growth arrest and DNA damage gene
GPx	Glutathione peroxidase
GSH	Reducing glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
$HClO_4$	Perchloric acid
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HNO_3	Nitric acid
hTERT	Human telomerase reverse transcriptase
ICP-MS	Inductive coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IDI	Iodothyronine deiodinase
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IMVS	Institute of Medical and Veterinary Science
K	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LDL	Low-density lipoprotein
Mg	Magnesium
MN	Micronuclei
MNed	Micronucleated
MT	Metallothionein
Mtase	Cytosine-5-methyltransferase
MTHFR	Methylenetetrahydrofolate reductase
MUFA	Monounsaturated fatty acid

Na	Sodium
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Sodium phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBud	Nuclear bud
NCEFF	National Centre of Excellence in Functional Foods
NDI	Nuclear division index
NHMRC	National Health and Medical Research Council
NOAEL	No observable adverse effect level
NPB	Nucleoplasmic bridge
8-OHdG	8-hydroxy-2-deoxyguanosine
P	Phosphorus
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PHGPx	Phospholipid hydroperoxide glutathione peroxidase
PROFORT	Wheat process-fortified with selenomethionine
PUFA	Polyunsaturated fatty acid
p-XSC	1,4-phenylenebis(methylene)selenocyanate
RDA	Recommended daily allowance
RDI	Recommended daily intake
ROS	Reactive oxygen species
S	Sulphur
SAM	S-adenosylmethionine
SARDI	South Australia Research and Development Institute
SCGE	Single-cell gel electrophoresis
SDG	Selenodiglutathione
SE	Standard error
Se	Selenium
SeAM	Se-adenosylmethionine
SECIS	Selenocysteine insertion sequence

SE-EMP	Selenium exchangeable metabolic pool
Sel	Selenoprotein
Se-met	Selenomethionine
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPS2	Selenophosphate synthetase-2
SSB	Single strand break
Top II	Topoisomerase II
TrxR	Thioredoxin reductase
UTR	Untranslated region
WAS	Waite Analytical Services
XPA	Xeroderma pigmentosum group A protein
Zn	Zinc

1. Literature Review: the Role of Selenium in Human Health and Genome Stability

1.1 Introduction

1.1.1 The element

The trace element selenium (Se) was first discovered in 1817 (Reilly, 1996). It has an atomic weight of 78.96 and its atomic number is 34. It belongs to group VIA, which also includes oxygen, sulphur and tellurium, and lies between arsenic and bromine in Period 4 in the periodic table.

1.1.2 Selenium distribution in soils

Selenium is one of the rarest elements in soils. It has a very uneven distribution globally with concentrations ranging from 0.01 mg/kg to 1200 mg/kg (Fordyce, 2005). Seleniferous areas include parts of the Great Plains of the USA and Canada, Enshi County in China, Colombia and Venezuela (Combs, 2001b; Gupta and Winter, 1975). In contrast, soils in New Zealand, Denmark, Finland, and north-east China are relatively low in Se, i.e. 0.1-0.6 mg Se/kg. Food plants take up this element from soil and thereby Se enters the food supply of animals and humans.

1.2 Selenium in human health

1.2.1 Selenosis

Ingestion of excessive seleniferous plant foods is considered the major cause of selenosis, or Se poisoning in humans in some parts of the world, where people depend on local foods. One large-scale endemic selenosis in humans was reported in the early 1980s by Yang and colleagues in Enshi County of China (Yang et al., 1983). In this seleniferous region, the reported dietary intake of Se was 3.2-6.7 mg/day, which is 50 times greater than normal intakes. Severe selenosis presents with malodorous breath, skin lesions such as bullous and itchy dermatitis, hair and nail changes or loss, mottled teeth and neurological abnormalities (SCF, 2000).

1.2.2 Selenim deficiency

The first major incident of a human Se deficiency disease was discovered in Keshan County in north-eastern China in 1935 (Diplock, 1981). Keshan disease is a cardiomyopathy predominantly in children, young adolescents and young women, resulting from an estimated Se intake of around 10 µg/day from only local food supply (Tapiero et al., 2003). Although Se deficiency is the major contributory factor in Keshan disease, viral infection is also a complicating factor. This underlying condition could induce widespread myocardionecrosis resulting from an impaired antioxidant system and increase the histopathological damage to the heart caused by Coxsackie virus, which has been isolated from patients suffering from the disease (Beck, 1997; Levander and Beck, 1997).

Kaschin-Beck disease is another endemic condition believed to be Se-related, which occurs in the low-Se regions with iodine deficiency in China and the former Soviet Union (Sokoloff, 1987). It is an osteoarthropathy, is characterised by a chronic disabling degeneration and necrosis of the joints and epiphysial-plate cartilages of the arms and legs. Typical symptoms include pain, stiffness and enlargement of joints and deformity of limbs. The condition may lead to growth retardation, and stunting was observed in affected children (Zhai et al., 1990). Myxoedematous cretinism may also result from combined iodine and Se deficiency (Allander, 1994; Moreno-Reyes et al., 1998; Moreno-Reyes et al., 2001; Sudre and Mathieu, 2001; Suetens et al., 2001).

In patients receiving long-term total parenteral nutrition, skeletal problems may develop because of inadequate intake of Se from the infusion fluids (Kajiyama et al., 2001; Kuroki et al., 2003; Reimund et al., 2000). Symptoms include myalgias and myopathy in some patients and cardiomyopathy in other patients when sepsis and/or vitamin E deficiency occurs. Selenium deficiency potentially could make the myocardium more susceptible to attack by free radicals (Reilly, 1996).

1.2.3 Suboptimal selenium status and related health conditions

Pure overt deficiency in humans is relatively rare now due to a mixed diet and food regulation in those areas (Yang et al., 1984). However, suboptimal Se status or inadequate dietary intake of Se has been recently implicated in a wide range of ill-health conditions/chronic diseases, such as cancers, cardiovascular disease, immunosupression and increased viral infection, increased oxidative stress and inflammation, impaired

thyroid hormone metabolism, infertility and impaired cognition/mood (Combs and Gray, 1998; Neve, 2002; Rayman, 2000a).

1.2.3.1 Cancer

Observational studies suggest an inverse association between low Se status, as indicated by toe nail Se concentration or blood Se concentration, and the risk of various cancers, particularly prostate cancer (Hartman et al., 2002; Nomura et al., 2000; Ujiie and Kikuchi, 2002; van den Brandt et al., 2003; Wei et al., 2004; Yoshizawa et al., 1998; Zeegers et al., 2002). However, there remains some inconsistency. There was no association found between serum Se status and lung or prostate cancer risk in a US population who were replete in Se (Goodman et al., 2001). Some Se supplementation studies supported the protective effect of Se in lung and prostate cancer; however, it was essentially limited to populations with low baseline Se levels (Duffield-Lillico et al., 2003; Reid et al., 2002).

Cancer is a condition with complex etiologic factors. Mechanisms for Se anti-cancer action are not fully understood. Animal studies suggest that Se has a protective effect at various stages of carcinogenesis (El-Bayoumy, 2001; Ip et al., 2002). Some of the hypotheses have been designed to explain the role of Se in cancer prevention, reflecting the key part that Se plays in cell metabolism and human health, such as antioxidant protection involving selenoenzymes, specific Se metabolites that discharge antitumorigenic functions, enhanced carcinogen detoxification, enhanced immune surveillance, modulation of cell proliferation, inhibition of tumour cell invasion and inhibition of angiogenesis (Cho et al., 2004; Combs and Gray, 1998; El-Bayoumy and Sinha, 2005; Lu and Jiang, 2005; Rayman, 2005).

1.2.3.2 Immunocompotence and infectious diseases

Se has a multi-factorial influence on both the innate and the acquired immune systems. A deficiency in Se inhibits leukotriene B4 synthesis in macrophages, neutrophil function, antibody production and proliferation of lymphocytes in response to mitogens (Arthur et al., 2003; Cao et al., 1992; Ito et al., 1998; Kiremidjian-Schumacher and Stotzky, 1987). These may collectively impair the cell-mediated immunity to kill invading foreign organisms and response to viral infection in humans when Se is deprivated (Kiremidjian-Schumacher et al., 1994). In a Se-depleted host, harmless viruses can become virulent when there is a reduction in functional GPx-1, as in the

case for the Coxsackie virus involved in Keshan disease (Beck, 1997; Beck et al., 2003; Nelson et al., 2001). Suboptimal Se status may increase the susceptibility of individuals to viruses such as hepatitis, influenza and HIV and further implicate in the induction of cancers, such as hepatocellular carcinoma due to the hepatitis B virus (Diamond et al., 2001; Taylor et al., 2000; Yu et al., 1999; Yu et al., 1997).

Supplementation studies showed that increased plasma Se concentration can improve the cellular immune response through an increased production of interferon gamma (IFN-γ) and other cytokines, enhanced activation and proliferation of B-cells, an earlier peak in T cell proliferation, an increase in T helper cells and the activity of natural-killer-cells, even in Se-replete individuals (Hawkes et al., 2001; Kiremidjian-Schumacher and Roy, 1998; Roy et al., 1994; Roy et al., 1995). Adults with marginal Se status showed more rapid clearance of the poliovirus after supplementation with sodium selenite for 15 weeks (Broome et al., 2004).

1.2.3.3 Cardiovascular disease

Suboptimal Se status has long been considered as a risk factor for atherogenesis. Earlier studies showed that low serum Se concentration was associated with increased risk of cardiovascular disease and higher mortality from the disease (Kardinaal et al., 1997; Salonen et al., 1982). A recent meta-analysis reported that Se concentration was inversely associated with coronary heart disease risk in observational studies (Flores-Mateo et al., 2006). The pooled data showed a 50% increase in Se concentration was associated with a mean reduction of 24% (7%-38%) reduction in coronary heart disease risk (Flores-Mateo et al., 2006). However, the findings from other randomized trials are still not conclusive. It seems that the association between Se and cardiovascular disease exists only at low Se status.

Se deficiency has been shown to be implicated in oxidative stress-mediated inflammatory and atherogenesis events (Prabhu et al., 2002; Brigelius-Flohe et al., 2003). In contrast, high serum Se status was shown to be predictive of a reduction in cyclooxygenase (COX)-mediated inflammation in men (Helmersson et al., 2005). A low Se status may also impair plasma GPx activity and contribute towards atherogenesis, mediated by platelet aggregation and plaque formation due to oxidation of low-density-lipoprotein (LDL) (Lee et al., 2003).

1.3 Human selenoproteins

In humans, the essentiality of Se was not fully established until the 1970s. Flohé et al. demonstrated that Se was an integral part of glutathione peroxidase in the form of covalently bound selenocysteine (Flohé et al., 1973; Sunde, 1997). Selenoproteins refer to Se-dependent proteins with endogenously synthesised selenocysteine used in ribosome-mediated protein synthesis, being specifically incorporated in their active sites (Korotkov et al., 2002). If it is replaced by its sulphur analogue cysteine, the activity of the enzyme is markedly reduced (Patching and Gardiner, 1999). Therefore, it is generally believed that Se exerts its biological functions in mammals mostly through selenoproteins, rather than the element *per se* (Ganther, 1999). There are at least 25 selenoproteins in humans (Kryukov et al., 2003). With advanced tools in selenoprotein research, such as ^{75}Se labelling experiments in combination with electrophoretic, chromatographic separation techniques and computer programs in screening of genome sequences, further selenoproteins are likely to be identified (Behne and Kyriakopoulos, 2001). However, among those that have been identified, only a few are well characterized. Selenoproteins with known functions are listed in Table 1-1 and those with poorly defined or unknown functions are listed in Table 1-2.

Table 1-1 Human selenoproteins with known functions

Selenoproteins	Functions
Glutathione peroxidises	
Cytosolic or classic GPx (cGPx, GPx-1)	A cytosolic antioxidant
Gastrointestinal GPx (GI-GPx, GPx-2)	Defence against ingested organic hydroperoxides; involved in apoptosis and proliferation regulation
Phospholipid hydroperoxide GPx (PHGPx, GPx-4)	A universal antioxidant in the protection of biomembranes; involved in redox signalling and regulatory processes; structural component of the sperm
Sperm nuclei GPx (snGPx)	Sperm maturation and male fertility
Iodothyronine deiodinases (IDI)	
Type 1 deiodinase (5'DI)	Converts T4 to T3, the major thyroid hormone in peripheral circulation; involved in the degradation of the inactive reverse T3 (rT3)
Type 2 deiodinase (5' DII)	Similar to 5'DI , the dominant form in brain
Type 2 deiodinase (5'DIII)	Inactivation of T3 and T4
Thioredoxin reductases	
Thioredoxin reductase 1 (TR1, TrxR1)	A cytoplasmatic enzyme involved in many aspects of cellular redox regulation
Testicular thioredoxin reductase (TR2, TrxR2)	A testis-specific enzyme located in the endoplasmic reticulum (ER)
Mitochondrial thioredoxin reductase (TR3, TrxR3)	Similar to TR1 but located in mitochondria
Selenophosphate synthetase-2 (SPS2)	Catalyses the production of selenophosphate for the synthesis of selenocysteine

(Behne and Kyriakopoulos, 2001; Birringer et al., 2002; Gromer et al., 2005)

Table 1-2 Human selenoproteins with poorly defined or unknown function

Selenoproteins	Possible function
Selenoprotein P (SelP)	Transport of Se; antioxidant; heavy metal detoxification
Selenoprotein W (SelW)	Involved in muscular function; redox metabolism
15-kDa selenoprotein (T cells) (Sel15)	Involved in the quality control of secreted proteins
18-kDa selenoprotein (Sel18)	Unknown
Selenoprotein H (SelH)	Unknown
Selenoprotein I (SelI)	Unknown
Selenoprotein K (SelK)	Unknown
Selenoprotein M (SelM)	Unknown
Selenoprotein N (SelN)	Involved in early development, proliferation and regeneration in striated muscles
Selenoprotein O (SelO)	Unknown
Selenoprotein R (SelR) (also referred as zinc-containing methionine-R-sulfoxide reductase 1, MsrB1, or as Selenoprotein X, Self)	Involved in numerous important biological processes, including antioxidant functions, regulation of enzyme activity and cell signalling, detoxification of sulfoxides
Selenoprotein S (SelS)	Involved in retrotranslocation of misfolded endoplasmic reticulum protein; interacts with inflammatory markers serum amyloid A 1β
Selenoprotein T (SelT)	Unknown
Selenoprotein V (SelV)	Unknown
Selenoprotein Zf1 (SelZf1)	Thioredoxin reductase homologues
Selenoprotein Zf2 (SelZf2)	Thioredoxin reductase homologues
Plasma GPx (pGPx, GPx-3)	Unknown
Glutathione peroxidase 6 (GPx-6)	Might have a function in olfaction

(Birringer et al., 2002; Behne and Kyriakopoulos, 2001; Gromer et al., 2005)

1.3.1 Selenoproteins in humans

1.3.1.1 Glutathione peroxidases (GPxs)

GPx-1 was the first specific mammalian selenoprotein identified (Schwarz et al., 1957; Schwarz and Foltz, 1957; Stadtman, 1980). Now, the family of GPx includes seven isoenzymes in humans; however, two of them (GPx-5 and GPx-7) are not selenoenzymes (Gromer et al., 2005). They exist in different parts of a cell or tissue using (typically) glutathione (R-OOH + 2GSH → R-OH + H_2O + GSSG), or other thiols to reduce a variety of organic hydroperoxides produced during normal cellular metabolism or upon exposure to environmental carcinogens, which otherwise damage the structure of macromolecules and consequently interfere with the functions of enzymes (El-Bayoumy, 2001). Therefore, they play an important role in the body's antioxidant defence system.

GPx-1, the classic GPx, is a cytosolic selenoenzyme and abundant in the liver and kidney (Behn et al., 1995; Dreher et al., 1997). It can metabolize a wide range of organic hydroperoxides but not fatty acid hydroperoxides in phospholipids (Arthur, 2000). Abnormality of GPx-1 expression may link to the etiology of cancers, cardiovasular disease, neurodegeneration, autoimmune disease and diabetes (Lei et al., 2007). GPx-2, or gastrointestinal GPx (GI-GPx), is found mainly within the gastrointestinal system, and also the human liver (Wingler and Brigelius-Flohe, 1999). It has similar substrate specificity to GPx-1 and may be the primary defence against the ingested organic hydroperoxides. GPx-2 could be involved in redox-regulation of apoptosis and proliferation (Brigelius-Flohe et al., 2001; Gromer et al., 2005). GPx-3 is a glycoprotein located extracellularly in the plasma and in the intestine. It has the second highest plasma concentration after selenoprotein P but its physiological function in plasma is not known (Arthur, 2000; Gromer et al., 2005). GPx-4 is capable of reducing all of the substrates other GPxs can and even phospholipid hydroperoxides integrated in membranes as well as cholesterol hydroperoxides in oxidised low density lipoprotein (Behne and Kyriakopoulos, 2001; Brown and Arthur, 2001). Thus, it may play a role as a universal antioxidant in the protection of biomembranes. GPx-4 could also be involved in redox signalling and regulatory processes, such as inflammation and apoptosis (Behne and Kyriakopoulos, 2001; Gromer et al., 2005). In the testes, GPx-4 transforms into oxidatively cross-linked, insoluble structural component of the sperm's

midpiece and may be essential for normal sperm morphology, motility and viability (Brown and Arthur, 2001; Neve, 2002). GPx 6 is expressed in olfactory epithelium and might have a function in olfaction, but this is yet to be proven (Arthur, 2000; Gromer et al., 2005). Sperm nuclei GPx, which is different from GPx-4, was detected only in testis and spermatozoa and might be necessary for sperm maturation and male fertility (Behne and Kyriakopoulos, 2001).

1.3.1.2 Deiodinases (5'DIs)

Three types of 5'DIs have been identified in mammals. This family of selenoproteins is responsible for the synthesis and degradation of thyroid hormones (Birringer et al., 2002). Thyroid hormones act primarily via intracellular receptors as transcription factors and are required for normal growth and development, thermogenesis and regulation of the basal metabolic rate.

5'DI-I is a plasma membrane protein. It regulates thyroid hormone activity by catalysing deiodinations of L-thyroxine (T4) – the major form secreted by the thyroid – to T3, which is the major active thyroid hormone in peripheral circulation. It is also involved in the degradation of the inactive reverse T3 (rT3) (Beckett and Arthur, 2005; Kohrle, 2005}. 5' DI-II is an endoplasmic reticulum (ER) membrane protein with functions similar to 5'DI. As the dominant form in the brain, 5'DI-II is responsible for more than 75% of the local T3 production (Gromer et al., 2005). The prime physiological function of 5'DI-III is to inactivate the thyroid hormones T3 and T4 (Behne and Kyriakopoulos, 2001; Kohrle, 2005).

1.3.1.3 Thioredoxin reductases (TrxRs)

The classical thioredoxin system consists of TrxRs and its associated substrate, the redox active protein thioredoxin (Trx) ($TrxS_2 + NADPH + H^+ \rightarrow Trx(SH)_2 + NADP^+$). Reduced Trx is reoxidized to provide reducing equivalents to various target molecules (Gromer et al., 2004). The thioredoxin/TrxR system has an unusual broad substrate spectrum and is involved in cellular and intercellular processes ranging from ribonucleiotide reduction to redox regulation of cytokine signalling (Birringer et al., 2002). Replacement of selenocysteine by cysteine reduces TrxR activity by 90% or more (Birringer et al., 2002).

Currently, three distinct human TxrRs have been identified. TxrR1 is a ubiquitous cytoplasmaic housekeeping enzyme and involved in many aspects of cellular redox regulation as well as in deoxyribonucleotide synthesis. TxrR2 is located in mitochondria with highest levels in the prostate and testis. Thioredoxin glutathione reductase or TxrR3, appears to be a testis-specific enzyme and is located in the ER (Gromer et al., 2005).

1.3.1.4 Selenophosphate synthetase 2 (SPS2)

SPS 2 catalyzes the formation of selenophosphate from selenide (HSe^- + ATP + H_2O → $HSePO_3^{2-}$ + HPO_4^{2-} + AMP), the assumed Se donor for the biosynthesis of selenocysteine (Gromer et al., 2005; Behne and Kyriakopoulos, 2001). The involvement of SPS2, itself a selenoprotein, in the biosynthesis of selenoprotein is of special interest with regard to the regulation of Se metabolism (Guimaraes et al., 1996).

1.3.1.5 Selenoprotein P (SelP)

SelP is among the first discovered selenoproteins but its function has not been fully understood. It is the major selenoprotein in plasma, which represents 44-70% of the total plasma Se (Hill et al., 1996; Patching and Gardiner, 1999). Human SelP contains 10 selenocysteine residues per polypeptide chain (McKenzie et al., 2002). Therefore, it may function primarily as a Se transport protein. SelP is synthesised mostly in the liver and released into blood circulation, then degraded to liberate Se for the synthesis of novel selenoprotein in target tissues. SelP may be required to retain Se and Se-dependent antioxidant system in the brain as it can also be expressed locally and not lost into general circulation (Scharpf et al., 2007). SelP may also function as a plasma antioxidant and heavy metal antidote as it contributes to the destruction of peroxynitrite, an important factor in inflammatory toxicity (Arteel et al., 1998).

1.3.1.6 Selenoprotein W (SelW)

SelW has been known for years as a protein enriched in skeletal and heart muscle, which was related to the ‘white muscle disease’ in Se-deficient lambs. It is also found widely distributed in other tissues, including brain, heart and kidney (Gu et al., 2000; Whanger, 2000). SelW is present in the cytosol as well as being membrane-associated. It has a potential function in muscular function and redox metabolism (Behne and Kyriakopoulos, 2001; Gromer et al., 2005).

1.3.2 Selenoprotein biosynthesis

The biosynthesis of selenoproteins is achieved by the synthesis and transfer of selenocysteine on a specific tRNA followed by specific co-translational insertion into protein. This multi-step process has some unique features. Firstly, the biosynthesis of endogenous selenocysteine always takes place on a selenocysteine-specific tRNA (Sec $tRNA^{[Ser]Sec}$), which is different from all other tRNAs. Secondly, the specific incorporation of selenocysteine into selenoproteins is encoded by the UGA codon in the respective mRNA, which normally serves as a termination signal. Thirdly, a hairpin secondary structure located mostly in the 3'-untranslated region (3'-UTR) of mRNA and specific translation factors are required to decode the codon for the selenocysteine insertion (Hatfield and Gladyshev, 2002). Figure 1-1 describes the biosynthesis of selenoproteins in humans.

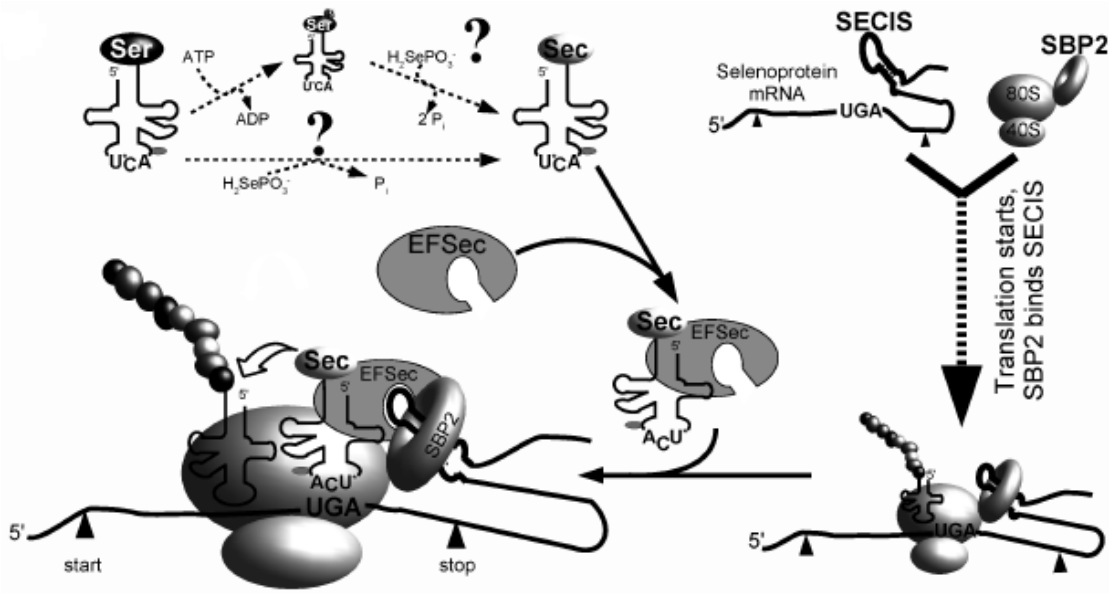


Figure 1-1 Simplified diagram of selenoprotein synthesis in humans (Gromer et al., 2005). SECIS, selenocysteine intersion sequence; EFSec, Sec-specific elongation factor; SBP2, SECIS-binding protein 2. Steps marked with dotted lines and question marks have not been fully elucidated. How the decision is made to cease translation is still not completely resolved.
Ser, serine; Sec, selenocysteine

1.3.2.1 Biosynthesis of selenocysteine on Sec $tRNA^{[Ser]Sec}$

The body maintains a low free concentration of the highly reactive compound selenocysteine (Levander and Burk, 1996). However, unlike other preformed amino acids, it cannot be used directly for loading its respective tRNA for selenoprotein synthesis (Allmang and Krol, 2006). The biosynthesis of selenocysteine is directed by

the RNA triplet UGA, also a stop codon, and always occurs on its tRNA (Hatfield and Gladyshev, 2002). A specific tRNA that decoded UGA as selenocysteine was identified (Lee et al., 1989). This Sec $tRNA^{[Ser]Sec}$ differs substantially from other 20 amino acid tRNA in size and shape. Long length in both D-stem and amino acid arm of the mammalian Sec $tRNA^{[Ser]Sec}$ is essential for aminoacylation (Allmang and Krol, 2006; Hatfield and Gladyshev, 2002). Methylation of the tRNA anticodon marks the final step in Sec $tRNA^{[Ser]Sec}$ maturation and is responsive to Se status (Diamond et al., 1993; Hatfield and Gladyshev, 2002).

Sec $tRNA^{[Ser]Sec}$ is initially aminocylated with serine by seryl-tRNA synthetase, which serves as the backbone for selenocysteine synthesis. This is followed by phosphorylation of the serine, and an intermediate between serine and selenocysteine is formed. The conversion of serine to selenocysteine on a Sec $tRNA^{[Ser]Sec}$ is completed when the active form of Se – assumed to be selenophosphate, formed by SPS2 using selenide with the consumption of ATP – is donated to the intermediate (Hatfield and Gladyshev, 2002). Alternatively, seryl-$tRNA^{[Ser]Sec}$ may transform into selenocysteinyl-$tRNA^{[Ser]Sec}$ directly by reaction with selenophosphate (Behne and Kyriakopoulos, 2001). However, both of the above mechanisms are not fully established in mammals.

1.3.2.2 Specific co-translational incorportation of selenocysteine

Newly formed selenocysteine is specifically incorporated into a protein through a co-translational process. There are several factors required to form a unique complex of translation components for the insertion of selenocysteine into protein besides the Sec $tRNA^{[Ser]Sec}$ and the in-frame UGA codon in selenoprotein mRNA. Recognition of UGA as a selenocysteine codon depends on secondary mRNA structure – the selenocysteine insertion sequence (SECIS) element (Allan et al., 1999). All selenoprotein mRNA contains at least one functional SECIS element, which resides in the untranslated regions (3'-UTR) in the vast majority of the cases but was found once in the 5' UTR (Allmang and Krol, 2006). The presence of the SECIS element dictates any in-frame UGA codon within the coding region to serve as selenocysteine when a minimal spacing requirement between UGA and SECIS element is met (Hatfield and Gladyshev, 2002). The element also functions by recruiting SECIS-binding protein (SBP2) and together with selenocysteine-specific elongation factor (Effect) to form a ribosomal complex during the co-translational selenoproteins synthesis, which is

required to compete with release factor in usual protein synthesis (Hatfield and Gladyshev, 2002).

1.3.3 Regulatory aspects of selenoprotein synthesis

The regulation of selenoprotein synthesis appears to be tissue-specific via individual selenoprotein mRNA at the transcriptional or post-transcriptional levels. This is in response to Se availability as well as other factors such as biological conditions and environmental factors (Wingler et al., 1999).

In general, the efficiency of selenoprotein synthesis, relative quantities and maturation of Sec $tRNA^{[Ser]Sec}$ are responsive to nutritional Se status (Hatfield and Gladyshev, 2002). Therefore, inadequate Se supply usually results in reduced levels and activities of selenoproteins. The instability, or enhanced degradation of translated selenoprotein mRNA may be the main regulatory factor for the intracellular differential in the expression of the selenoproteins in Se deficiency (Allan et al., 1999; Behne and Kyriakopoulos, 2001; Muller and Pallauf, 2003). It has been shown that the expression of GPx-1 and SelW mRNA decreased dramatically in Se-depleted human and rat colon cells while less effect was observed in GPx-4 and GPx-2 mRNA abundance (Pagmantidis et al., 2005). However, during Se deprivation, GPx-4 mRNA and 5'D mRNA in the thyroid increased 50% and 90%, respectively (Allan et al., 1999).

The hierarchy in selenoprotein expression is an interesting phenomenon observed in mammals during the periods of insufficient dietary Se intake (Behne and Kyriakopoulos, 2001). This ensures that the Se levels in certain tissues are retained and within each tissue the levels of the metabolically most important selenoproteins maintained (Birringer et al., 2002; Daniels, 1996; Kohrl et al., 2000). During Se deprivation in the diets of rats and mice, the amounts of this element was substantially reduced in liver and kidney, while brain and testes preserved most of their Se (Hatfield and Gladyshev, 2002). The thyroid also retains Se in severe states of deficiency and all TrxRs rank highly in the hierarchy of selenoproteins (Birringer et al., 2002). The PHGPx and 5'DI are preferentially supplied with the element over the GPx-1 within most of the tissues when Se consumptions are limited (Allan et al., 1999; Villette et al., 1998; Wingler and Brigelius-Flohe, 1999).

1.4 Selenium metabolism in humans

1.4.1 Forms of Se in human diet

In human diet, two naturally occurring selenoamino acids make up the bulk of the element, i.e. selenomethionine (Se-met), the predominant form in food plants, and selenocysteine (Se-Cys), mainly found in food from an animal source. There are other organic selenocompounds present in very small quantities in human diet, such as Se-methyl selenocysteine, γ-glutamyl Se-methyl selenocysteine, selenocystathione, selenohomocysteine, γ-glutamyl selenocystathione and methyl selenol (Finley, 2005; Reilly, 1996). Selenite and selenate are two inorganic forms of Se that are often used as dietary supplements in humans.

1.4.2 Absorption and transportation

Selenium in foods is readily absorbed with apparently no physiological control over the absorption. In general, the absorption of the organic forms of Se is more efficient than that of inorganic forms, with uptake from the gastrointestinal tract of more than 90% of selenomethionine compared with about 60 % of selenite (Reilly, 1996; Finley, 2006; Finley et al., 2004). The uptake of selenomethionine appears to utilise the active transport process using the same system as methionine. Competition for uptake occurs between methionine and its seleno analogue. The inorganic Se and other organic forms of Se, including selenocysteine, are absorbed from the gastrointestinal tract via a normal concentration gradient, which is a passive process and competes with inorganic sulphur compounds for absorption (Daniels, 1996). Selenomethionine is initially incorporated non-specifically into tissue proteins, including red cell haemoglobin and plasma albumin, and transported around the body before being metabolised (Levander and Burk, 1996; Wolffram et al., 1989). When selenomethionine is the main form of the element in people's diet, nearly 50% of the Se in plasma is associated with albumin (Burk, 2002; Whanger et al., 1996).

1.4.3 Metabolism pathway

Depending on the chemical form and Se status, the ingested Se could follow different metabolic pathways in the body (Figure 1-2). In general, all absorbed Se would either be metabolised in the biologically active pool (termed 'the Se exchangeable metabolic pool, SE-EMP'), which provides the basis for metabolism and synthesis of all the functionally important selenoproteins and seleno-metabolites for excretion, or enter the

body Se-binding protein pool, which derives from non-specific incorporation of exogenous selenomethionine into tissue proteins in place of methionine. The later is considered as a Se store in the body (Daniels, 1996; Reilly, 1996).

Figure 1-2 The metabolic pathways of dietary forms of Se. Adapted from Rayman M, 2004 (Rayman, 2004)

Se-MeSeCys, selenomethyl-selenocysteine; Se-Cys, selenocysteine; Se-Met, selenomethionine; GS-Se-SG, selenodiglutathione; GS-SeH, selenoglutathione.

Inorganic Se, such as selenate and selenite, cannot be stored, but enters the reductive pathway. They react directly with glutathione to form selenodiglutathione, which is readily reduced to selenide by thiols and NADPH-dependent reductases such as thioredoxin reductase in mammals (Birringer et al., 2002; Finley, 2006; Ganther, 1999).

Se-met is activated initially by adenosylation, demethylated and converted to selenocysteine via selenohomocysteine and selenocystathionine, in analogy to methionine (Griffiths et al., 2006; Schrauzer, 2000). It can be converted into selenocysteine by trans-selenation reaction or transsulfuration pathways (Daniels, 1996). However, neither the selenocysteine that is produced in this way nor that absorbed from diet can be used directly for selenoprotein synthesis. Only after further

metabolism to selenide through the β-lyase reaction, this tissue-stored selenomethionine as well as the dietary selenocysteine is utilised for the synthesis of selenoproteins {Letavayova, 2006 #545; Reilly, 1996 #11}. In addition to the pathway via selenocystiene, selenomethionine can be converted to methylselenol directly through the reaction catalysed by the enzyme cystathionine γ-lyase, but this occurs only in the case of excessive dosage (Okuno et al., 2005a; Okuno et al., 2005b).

Methylated forms of Se, such as methyl-selenocysteine, cannot be accumulated in the body. Often, they are catalysed by β-lyase to form monomethyl-selenol thereby entering the methylation pathway for further methylation and excretion (Foster et al., 1986b; Foster et al., 1986a; Ip et al., 1991). However, monomethyl-selenol could also be available for selenoprotein synthesis through a demethylation process to form selenide (Ip et al., 2002).

Selenide is a key metabolite in the utilisation and methylation excretion of Se. It can be converted to selenophosphate involved in the synthesis of selenocysteine on Sec $tRNA^{[Ser]Sec}$ and functional selenoproteins (Daniels, 1996; Ganther, 1999), or be methylated by S-adenosylmethionine-dependent methyl transferase and generate excretory metabolites when there is surplus Se in the SE-EMP (Birringer et al., 2002).

1.4.4 Storage and excretion

The Se store in body comprises Se-binding proteins and selenoproteins. Humans cannot synthesize selenomethionine, therefore body store of Se is proportional to its intake (Reilly, 1996). In mammals, Se preferentially accumulates in the endocrine glands, brain and reproductive organs (Allan et al., 1999). When dietary Se supply is interrupted, turnover of the selenomethionine pool provides Se to the organism (Levander and Burk, 1996). Absorbed selenomethionine can substitute for methionine in tissue proteins and be reutilized (Daniels, 1996; Finley, 2006). Selenomethionine is not known to have a physiological function separate from that of methionine (Allan et al., 1999; Levander and Burk, 1996). When methionine supply is adequate or dietary Se supply is interrupted, Se released from degraded selenomethionine is available to contribute to the Se exchangeable metabolic pool (Daniels, 1996; Sunde, 1997).

Selenium is excreted mainly by three distinct routes, in urine via the kidneys, in faeces from the gastrointestinal tract and in expired air via the lungs. The urine pathway

appears to be the dominant excretion route for Se in humans, which regulates the Se levels in the body (Reilly, 1996). The Se metabolites in human urine is in the form of selenosugars, 1-β-methylseleno-*N*-acetyl-D-galactosamine (Kobayashi et al., 2002; Kuehnelt et al., 2005), the reaction products of glutathionine conjugated selenide and the reactive sugar moiety followed by methylation, and the very soluble trimethylselenonium (Suzuki et al., 2006). Faecal Se consists largely of unabsorbed dietary Se, along with Se contained in biliary, pancreatic and intestinal secretions. Excretion of Se via the pulmonary route in expired air in the form of the volatile dimethylselenide occurs principally when intake is unusually high. The garlic-like odour of this compound on the breath is characteristic of Se intoxication (Reilly, 1996). Generally, the methylation pathway is considered to be the detoxification pathway for all Se in the diet or in supplements (Lu et al., 1995).

1.5 Assessment of Se status in humans

1.5.1 Tissue concentration – the primary biomarker of Se status

Tissue concentration of Se is a primary biomarker of status of the element in humans. Blood Se concentration has been widely used as an indicator of Se status in both clinical practice and epidemiologic research (Sunde, 1997). Plasma Se levels respond to Se supplementation as early as 5 days (Levander et al., 1983a; Neve et al., 1988) and can therefore reflect short-term Se status. Plasma Se provides a good index of the severity of impaired Se status. However, plasma Se continues to increase when intake from selenomethionine is high and therefore, may not reflect true Se status since a large proportion of absorbed selenomethionine is incorporated into proteins. Selenium in hair and toenail may provide certain useful insights into the long-term status. However, the use of shampoo and nail polish containing this element by people in Western countries may limit the use of these sample materials (Xia et al., 2000).

1.5.2 GPx activity – the functional biomarker of Se status

It is suggested that a better measure of Se status should reflect the amount of Se that is available for activity of the functional selenoproteins (Neve, 1995; Thomson et al., 1993). GPx activity is one of such useful indicators of Se status and bioavailability and bioefficacy of Se (Sunde, 1997). This functional parameter responds to Se supplementation in only one or two weeks (Levander et al., 1983b; Thomson et al., 1993; van der Torre et al., 1991), and therefore, provides a sensitive measure of Se

status in population groups with relatively low Se intake. However, the GPx activity reaches a plateau once the blood Se concentration reaches 100 µg/l (Daniels, 1996; Hambidge, 2003; Sunde, 1997). Platelets have been shown to be reliable for assessing short-term Se status (life span 8-14 days in humans) (Levander et al., 1983a). They are rich in GPx and do not contain GSH-S-transferase and haemoglobin, which has some intrinsic peroxidase activity (Reilly, 1996). Also, platelet GPx appears to plateau at higher plasma Se concentrations compared to red blood cell GPx (Thomson, 2004a). Selenoprotein P and deiodinases and T3/T4 ratio are also functional biomarkers of selenium (Arthur, 1999; Elsom et al., 2006).

1.6 Dietary sources of Se in humans

1.6.1 Dietary sources of Se

Selenium is found in a wide range of foods. The Se content of foods varies significantly, reflecting the level of the element in the soil in which they grow or the Se content in feed on which they are fed. Therefore, different foods have various Se contents and the Se level in the same food would show regional variation (Reilly, 1996).

Good dietary sources of Se are protein-rich foods, such as meat, poultry and fish. Se levels in various food groups in Australia are shown in Table 1-3. Bread and cereals are of particular importance for dietary Se intake in the Western diet (Lyons et al., 2003a). Even though the actual concentration may not be very high in bread and cereals, the frequency and quantity of consumption of these foods is sufficient to make it a major source of the element for many people (Lyons et al., 2003a). It is the second most important source of dietary Se in the USA and in the UK, and has been found to supply almost a third of the daily Se intake of Australian children (Barrett et al., 1989; Lyons et al., 2003a).

Table 1-3 Selenium level in some food groups in Australia

Food group	Selenium (µg/g wet weight)
Cereals, cereal products	0.01 – 0.31
Meat, chicken, eggs	0.10 – 0.30
Raw fish	0.30 – 0.70
Milk, dairy products	0.01 – 0.11
Vegetables, fruit	0.002 – 0.01

* Data reported by Tinggi U et al. (Tinggi et al., 1992).

The richest dietary source of Se is Brazil nuts, which are produced by *Betholletia excels*, a Se accumulator (Reilly, 1996). The Se content in Brazil nuts (per 100g edible Brazil nuts) can vary from 2960 µg in US, 20-1500 µg in Australia to 5-530 µg in UK. Other foods that are rich in Se are kidney (127 – 149 µg/100g) and liver (82 µg/100g) (US data) (Lyons et al., 2003b). However, these foods are unlikely to be consumed in large quantities.

The most widely used techniques for measuring of Se content and different Se species in foods are inductive coupled plasma mass spectrometry (ICP-MS) and inductive coupled plasma-optical emission spectrometry (ICP-OES) after digestion of samples with acids, such as nitric acid (HNO_3) or nitric and perchloric acid ($HNO_3/HClO_4$) (Sturup et al., 2005; Zarcinas, 1984). However, they are not sufficiently sensitive to allow the routine and rapid determination of different species of Se at the low levels found in the majority of foods (Reilly, 1996). The use of elemental mass spectrometry and molecular mass spectrometry in combination is suggested for elemental speciation analysis (Dumont et al., 2006).

1.6.2 Bioavailability and bioefficacy of dietary Se in foods

Bioavailability of a nutrient can be broadly defined as including absorption and utilization of nutrients, which may be affected by the form of the nutrient and the body's requirement (Fairweather-Tait, 1997a and 1997b). Bioefficacy of a nutrient refers to the proportion of the ingested nutrient converted to an active form of the nutrient in body and therefore, a biological function of bioavailability (Brouwer et al., 2001). The major forms of Se in the human diet, namely selenomethionine, selenocysteine, selenite and selenate, are highly bioavailable to correct Se deficiency, raise plasma Se concentration, and are bioefficacious in restoring selenoprotein level and activity (Levander et al., 1983; Xia et al., 1989; Thomson et al., 1993; van de Torre et al., 1991; Brown et al., 2000; Al-Taie et al., 2003; Xia et al. 2005).

Selenomethionine, usually delivered as high-Se wheat or selenised yeast, is one of the most bioavailable and bioefficacious forms of Se. It seems to be a better form of Se for long-term maintenance of blood Se status and selenoprotein activities due to the non-specific incorporation of selenomethionine into tissue proteins (Finley, 2006). Selenomethionine was shown to be more effective in raising plasma Se levels than was selenite in both Se deficient and Se replete subjects in a dose-dependent manner (Burk

et al., 2006; Meltzer et al., 1992; Thomson et al., 1993; Xia et al., 2005). It has been shown in intervention studies that elevated GPx activities remained higher than baseline level in the group fed on selenomethionine (or hight-Se wheat) after supplementation ceased for ten weeks (Levander et al., 1983a; Thomson et al., 1985; van der Torre et al., 1991).

1.7 Selenium requirement for human health and current status/ intake of Australians

1.7.1 Selenium requirement for human health

The minimum Se requirement is determined by comparing dietary intakes in geographical areas in which deficiency occurs with intakes in those areas without such deficiency. As indicated by the dietary intake in areas where Keshan disease is not found, at least 19 and 14 µg/day for adult males and females, respectively, is needed for prevention of Keshan disease (Yang et al., 1988).

Graded supplementation studies in Se depleted subjects or those low in Se intake, in conjunction with GPx measurements have been used to assess the physiological requirement of Se at which plateau activities are achieved. Chinese investigators found that dietary intake of at least 41 µg Se/day would be nutritionally adequate to support full expression of selenoprotein GPx and their activities in plasma based on an early study in subjects already depleted in Se (Levander and Whanger, 1996; Yang et al., 1988). A similar study by a group of New Zealand investigators showed that 39 µg/day was sufficient to reach two-thirds of maximal GPx activity. The upper estimated Se requirement was calculated as 90 µg/day for maximal plasma GPx activity (Duffield et al., 1999). The level would result in a plasma Se concentration of 70 - 100 µg/l for maximizing GPx activities (Duffield et al., 1999; Neve, 1991; Neve, 1995; Thomson, 2004a). Adjustment for body weight and population characteristics led to establishment of a dietary recommendation of Se in several countries based on these human data. In Australia, current recommended dietary intake (RDI) of Se is 70 and 60 µg/day for adult men and women (National Health and Medical Research Council, 2006) using the plateau of GPx and selenoprotein P expression as the criterion (Duffield et al., 1999; Yang and Xia, 1995). However, the dietary standard for Se released by the World Health Organization (WHO) is lower because this group felt that only two thirds of the maximal GPx activity was necessary for its normal physiological function and

recommended a daily dietary intake in the range of 21-40 µg Se/day (WHO, 1996). The UK reference nutrient intake is 75 µg Se/day for adult men and 60 µg Se/day for adult women. The U.S. recommendation is 55 µg Se/day for both genders (Jackson et al., 2003).

Combs considered that the limited expression of selenoenzymes would constitute a subclinical deficiency of the element (Combs, 2001a). Emerging evidence indicates that a supra-nutritional intake of Se may give additional health benefits in Se-replete subjects for improving functions of the immune system and prevention of certain human degenerative diseases, such as cancer (Kiremidjian-Schumacher et al., 1994; Clark et al., 1996; Rayman, 2000 and 2002). It has been also suggested that in Se replete subjects, 300 µg Se/day (diet plus supplement) and plasma Se of 120 µg/l may be required to significantly reduce cancer risks (Clark et al., 1998; Combs et al., 2001; Duffield-Lillico et al., 2002). However, these levels associated with anti-carcinogenesis are at least 10-fold those required to prevent clinical signs of deficiency and well above that required for maximizing selenoprotein functions.

1.7.2 Selenium status/intake of Australians

There is insufficient data on Se status or intake in Australians to determine the extent of deficiency. According to a 1989 study, Australian dietary intake of Se is 57-87 µg/day (Reilly, 1996), which is comparable to current RDI and being moderate nationwide (Rayman, 2005; Thomson, 2004b). Based on previous published Australian studies (post-1990), the mean plasma Se level was about 89 µg /l (Lyons et al., 2004c). A Se status survey in blood donors of Adelaide, South Australians conducted in 2002 suggested a mean plasma Se concentration of 103 µg/l (Lyons et al., 2004a). Using plasma Se level of 70 µg /l as the minimum level for maximisation of GPx activities (Neve, 1995), it appears that Australians are ingesting sufficient Se to meet this physiological requirement.

However, when compared with early data, Lyons et al. suggested that there was an apparent decline in Se status from the late 1970s (mean plasma Se 122 µg/l, estimated from whole blood Se) to the late 1980s (mean plasma Se 91 µg/l). This could be explained by a decline of Se in the Australian food supply, particularly in wheat, due to more intensive crop production, soil acidification and the use of high sulphur fertilisers which displaces Se (Lyons et al., 2004a).

1.7.3 Strategy for increasing Se intake

An increased intake of Se is likely to be beneficial for populations who have inadequate Se intake or with higher Se requirements. For example, smokers, pregnant or lactating women, the frail elderly, and patients with HIV/AIDS may have Se requirements higher than average for optimizing the protective effects of selenoproteins and minimizing undesirable risks of degenerative diseases (de Jong et al., 2001; Golubkina and Alfthan, 2002; Lyons et al., 2004b).

Human Se status is mainly determined by dietary Se, and is, therefore, sensitive to increased Se content in the food supply. (Robberecht and Deelstra, 1994). It has been suggested that "agronomic biofortification is a food system approach that can deliver increased Se to a whole population safely, effectively, efficiently and in natural organic forms" (Graham et al., 2001). Food crop biofortification is a natural way of increasing micronutrient contents and in the case of Se, it is possible to apply Se fertilisation in food crops via soil and/or leaves. Crops with enhanced Se uptake efficiency then produce a higher level of Se in their edible parts, such as high Se wheat grains (Lyons et al., 2004b). This approach had been practised successfully in Finland, where the use of Se-enriched fertilisers has resulted in a doubling of dietary intake of the element (Aro et al., 1995). Biofortified foods are considered as functional foods since they may provide health benefits beyond the traditional nutrients they contain and have "functional" properties (Patch et al., 2004).

1.8 Selenium toxicity and reference values

1.8.1 Selenium toxicity

Toxicity of Se depends not only on the chemical form and quantity of the element consumed, but also on individual variations such as age, physical state, live weight, nutrition and diet of the person (Reilly, 1996). Both organic and inorganic forms of Se are toxic at high dose. Organic selenocompounds, such as selenomethionine, are usually lower in acute toxicity than inorganic selenocompounds as they are mostly food-bound and incorporation into tissue proteins removes a significant portion from circulation, thereby attenuating the adverse effects. However, excessive inorganic Se that cannot be used directly for selenoprotein synthesis must remain in circulation until it can be methylated and excreted. It has been shown in pigs that the toxicity difference between

selenite and selenomethionine diminished at dietary Se levels above 5 mg/kg (Schrauzer, 2000; Schrauzer, 2003).

The biochemical basis of Se toxicity at high levels of intake is not fully understood. It is possible that excess substitution of methionine residues with selenomethionine may alter the physiochemical properties of structural protein (and the activity of enzymes) because the seleno moiety of selenomethionine is more hydrophobic than the thiol moiety of methionine (Hatfield and Gladyshev, 2002; Schrauzer, 2003). The formation of hydrogen selenide extracellularly may be the key event in selenocompound metabolism that induces oxidative stress and toxicity through overt consumption of intracellular reduced glutathione (Gopalakrishna et al., 1997; Spallholz, 1994; Tarze, et al., 2007). It is also possible that Se interferes with enzyme activity, particularly by catalytic oxidation of sulphyhydryl (thiol) groups involved in oxidative metabolism within cells and thereby interfere with biosynthesis of essential molecules and DNA repair (Levander and Burk, 1996; Reilly, 1996; Hartwig et al., 2002).

1.8.2 Upper limit (UL) reference values

Selenium toxicity data in humans are limited. The lowest level of Se intake in Chinese living in a region naturally high in Se without overt poisoining was 910 µg/day, and no evidence of selenosis was seen in US adults consuming as much as 724 µg/day (Hathcock, 2004; Reilly, 1996; Yang and Zhou, 1994). Based on these observations, the no observable adverse effect level (NOAEL) was established for intakes of 800 µg/day and plasma Se 1000 µg/l (Food and Nutrition Board, 2000). However, in some sensitive individuals, the maximum safe dietary intake may be as low as 600 µg /day (Rayman, 2000b). In Australia, the upper level of intake for Se is suggested to be 400 µg/day for adults of 70 kg body weight, corresponding to one half of the NOAEL (National Health and Medical Research Council, 2006). Safety levels of Se intake set by different authorities are listed in Table 1-4 (Hathcock, 2004; National Health and Medical Research Council, 2006; Schrauzer, 2003).

Table 1-4 Selenium upper levels of intake for adults set by different authorities.

Authority	µg/day
Australia, NHMRC (UL)	400, 70kg body weight
US, EPA (RfD)	350, 70kg body weight
UK, EVM (UL)	450
European Commission, SCF (UL)	300

NHMRC – National Health and Medical Research Council; UL – upper limit of intake or tolerable upper intake level; SCF-Scientific Committee on Food; EVM- Expert Group on Vitamins and Minerals; EPA-Environmental Protection Agency.

1.9 Genome instability/damage in human health

1.9.1 Genome instability/damage and degenerative diseases

Genomic instability or genomic damage is a term used to describe a phenomenon that is the accumulation of the multiple genetic or chromosomemal changes required to convert a stable genome of a normal cell to an unstable genome characteristic of a tumour. Genome instability is characterized by chromosomal rearrangements and aberrations, amplification of genetic material, aneuploidy, micronucleus formation, microsatellite instability, altered methylation of CpG sequences and gene mutation (Smith et al., 2003).

Many factors have been identified that can compromise genome stability by inducing various types of DNA damage, including 1) environmental agents, such as UV light, X-rays, ionising radiation, diet and genotoxic chemicals including cigarette smoke; 2) (by)products of normal cellular metabolism, such as excessive reactive oxygen species (ROS); 3) spontaneous disintegration of some chemical bonds in DNA, such as deamination and hydrolysis of nucleotide residues (Hoeijmakers, 2001). Common DNA damages are uracil mis-incorporation, abasic site, 8-oxoguanine, single strand break (SSB), 6-4 photoproduct, cyclobutane pyrimidine dimer, bulky adduct, double strand break (DSB), inter-strand cross-link, A-G/T-C mismatch, DNA insertion and deletion resulting from inefficient DNA repair mechanisms leading to elevated DNA damage and increased levels of genomic instability. DNA mis-repair and accumulation of DNA damage is considered to be the direct cause of genome instability.

Mammalian cells have many mechanisms to eliminate DNA damage (Figure 1-3) (Friedberg, 2003; Hoeijmakers, 2001). Firstly, the antioxidant defence system can

significantly reduce ROS and protect DNA from oxidative stress induced damage. Secondly, DNA repair systems are crucial to minimise DNA damage and guarantee the stability or proper function of genes (Lindahl and Wood, 1999). Base-excision repair, nucleotide-excision repair, recombinational repair and mismatch repair are the common DNA repair mechanisms in eukaryotic cells. Thirdly, in the case of severe genome damage, DNA transcription, replication, chromosome segregation will be inhibited and cell division arrested, and the cell may finally initiate apoptosis. If the damaged cells survive, then mutant cells accumulate, which are considered to be the fundamental cause of many degenerative diseases including cancer. However, excessive cell death is also pathological in non-proliferating tissues such as brain, muscle and heart. Degeneration in this context implies the deterioration of the physical state or change of tissue from a higher to a lower functionally active form which is below normal and possibly pathological.

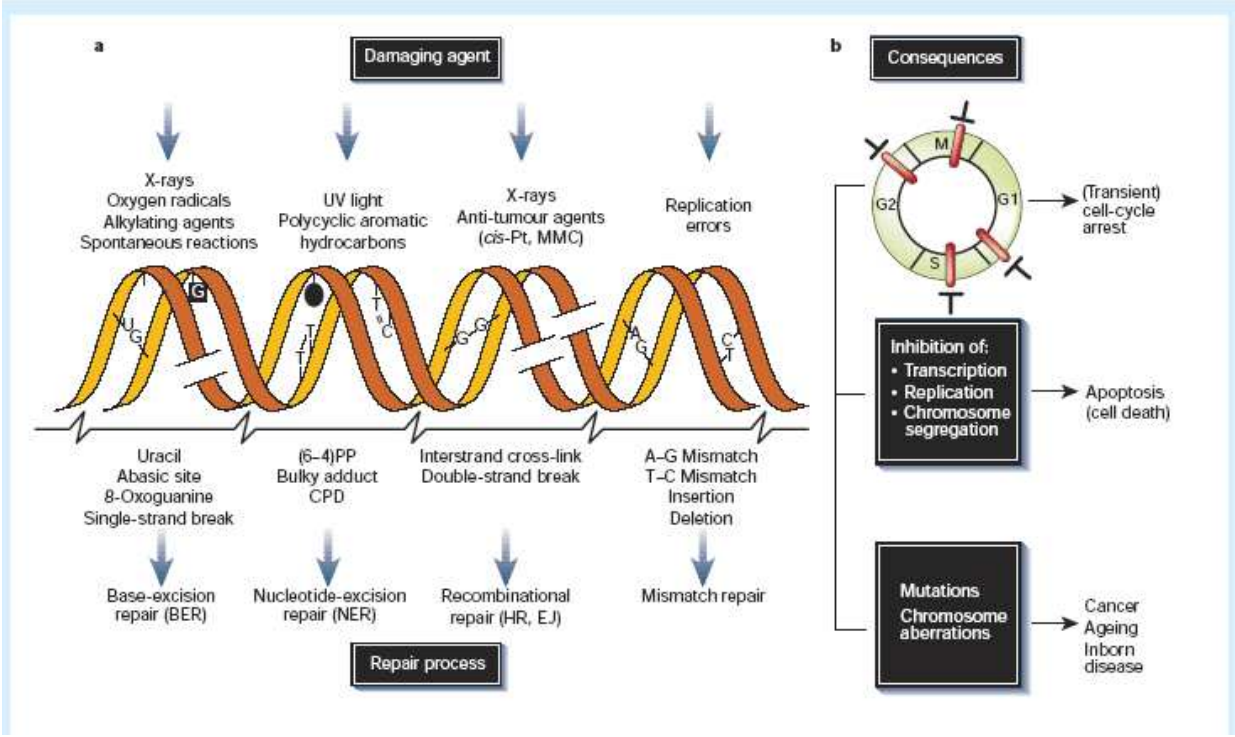


Figure 1 DNA damage, repair mechanisms and consequences. **a**, Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom). **b**, Acute effects of DNA damage on cell-cycle progression, leading to transient arrest in the G1, S, G2 and M phases (top), and on DNA metabolism (middle). Long-term consequences of DNA injury (bottom) include permanent changes in the DNA sequence (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects. Abbreviations: *cis*-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining.

Figure 1-3 DNA damage, repair mechanisms and consequences.

* From Genome maintenance mechanisms for preventing cancer (Hoeijmakers, 2001)

Genome instability has been linked to degenerative diseases and other adverse health outcomes, such as cancers, premature ageing and dementia, and congenital birth defects (Fenech, 2003). For example, at least three forms of genomic instability have been identified in colon cancer (Grady, 2004). The loss of tumour suppressor gene (*BRCA1* or *BRCA2*) function was usually found in breast cancer cells (Futreal et al., 1994). In prematurely ageing individuals, oxidative DNA damage increased markedly in some genes with reduced expression (gene silencing) in aged cortex (involved in learning, memory and neural survival) so initiating brain ageing after 40 years of age (Lu et al., 2004). Higher rates of micronuclei (MN) frequency, a biomarker of DNA damage, are exhibited in patients with Alzheimer's disease and Parkinson's disease (Petrozzi et al., 2001; Petrozzi et al., 2002).

1.9.2 Genome instability/damage and micronutrient status

Many micronutrients (vitamins and minerals) are involved in aspects of DNA maintenance. They are required, as substrates and/or co-factors, for DNA sythesis and repair, DNA methylation, prevention of oxidative damage to DNA or normal enzyme function (Table 1-5). Sub-optimal micronutrient status for DNA maintenance can cause DNA damage to an extent that is similar to that induced by other genotoxic agents, such as chemical carcinogens or ionising radiation (Fenech, 2003). This has been demonstrated, using the cytokinesis-block micronucleus (CBMN) cytome assay which showed an increase of micronucleus frequency under condition of folate deficiency within the physiological range (Fenech, 2001).

Genome/DNA damage can therefore serve as a sensitive biomarker of sub-optimal (inadequate or excessive) micronutrient status and may be predictive of disease risk before clinical consequences are manifested (Fenech, 2003). Optimal intake level of essential nutrients/minerals, is based on the definition of the 'total dose-response curve' of these substances. The bell-shaped curve describes biological function as depending on the degree of exposure such that both deficiency or excess may cause toxicity (Mertz, 1998). Multiple genome damage endpoints may be required to determine an optimal dosage of a micronutrient for minimizing genome instability.

Table 1-5 Role of micronutrients in DNA maintenance and consequence of deficiency

Micronutrients	Role in DNA maintenance	Consequence of deficiency
Vitamin A	Bind to RAR or RXR and interact with gene transcription factors, activate or inhibit gene expression	Potentially disrupt normal gene expression
Vitamin E, C	Prevention of oxidation to DNA and lipid oxidation	Increase baseline level of DNA strand breaks, chromosome breaks and oxidative DNA lesions and lipid peroxide adducts on DNA
Folate, vitamin B_2, B_6, B_{12}	Maintenance of DNA methylation; synthesis of dTTP from dUMP and recycling of folate	Uracil misincorporation in DNA, increased chromosome breaks and DNA hypomethylation
Niacin	Required as substrate for PARP which is involved in cleavage and rejoining of DNA and telomere length maintenance	Increased level of unrepaired nicks in DNA, increased chromosome breaks and rearrangements, and sensitivity to mutagens
Zn	Required as a cofactor for Cu/Zn superoxide dismutase, endonuclease IV, function of p53, Fapy glycosylase and in zinc finger proteins	Increased DNA oxidation, DNA breaks and elevated chromosome damage rate.
Fe	Required as component of ribonucleotide reductase and mitochondrial cytochromes	Reduced DNA repair capacity and increased propensity for oxidative damage to mitochondrial DNA
Mg	Required as cofactor for a variety of DNA polymerases, in DNA repair mechanisms. Essential for microtubule polymerisation and chromosome segregation	Reduced fidelity of DNA replication. Reduced DNA repair capacity. Chromosome segregation errors
Mn	Required as a component of mitochondrial Mn superoxide dismutase	Increased susceptibility to superoxide damage to mitochondrial DNA and reduced resistance to radiation-induced damage to nuclear DNA
Vitamin D	Prevention of oxidation to DNA and lipid oxidation. Induce apoptosis in cancer cells Involved in synthesis of oncogene, polyamine and calcium-binding proteins	Unknown
Polyphenols	Inhibition of topoisomerase	Unknown May induce chromosome breakage/ rearrangement and initiate cancer or kill cancer cells
Cu	Required as cofactor for cytochrome c oxidase, Cu/Zn superoxide dismutase, seruloplasmin, tyrosinase, lysyl oxidase, domaine-monooxygenase and peptidylglycine α-amidating monooxygenase	Increased DNA oxidation, DNA breaks and elevated chromosome damage rate.

Adapted from Fenech, 2003 and Fenech and Ferguson, 2001 (Fenech, 2003; Fenech and Ferguson, 2001).

RAR/RXR, retinoic acid receptors; PARP, poly(ADP-ribose)polymerase; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate.

1.9.3 Assessment of genome damage in lymphocytes

Human peripheral blood is a uniquely accessible tissue for examining genome damage in volunteers in a clinical setting, and is suitable for both *in vitro* and *in vivo* studies. Circulating lymphocytes continuously maintain a vigilant and comprehensive surveillance of the body for signs of infection or other threats. DNA damage in circulating lymphocytes can potentially provide an early warning of stresses they are exposed to, which is useful in predicting *in vivo* effects and cancer risk in humans (Bonassi et al., 2000). The CBMN cytome assay and the Comet assay are two popular measures used for assessment of genome damage at the cellular level.

1.9.3.1 The cytokinesis-block micronucleus cytome (CBMN Cyt) assay

The CBMN Cyt assay is currently one of the most widely used, comprehensive, and sensitive tests for direct and/or indirect measurement of various aspects of cellular and nuclear dysfunction in dividing cells. It is used widely in the pharmaceutical industry as the gold standard of genotoxicity testing, in human biomonitoring of environmental exposures, preventive medicine and nutritional efficacy (Fenech, 2007). It has also been shown to be a sensitive measure under conditions of elevated oxidative stress and genomic hypomethylation (Fenech, 2001). It has been validated as a biomarker of genome damage, aging and nutritional deficiency or excess (Courtemanche et al., 2004; Fenech, 2006). The genotoxicity and cytotoxicity events measured by this assay include DNA damage and misrepair, chromosomal instability (i.e. breakage, loss and rearrangement), gene amplification, mitotic abnormalities, cell death (i.e. necrosis and apoptosis) and cytostasis or cell proliferation (Fenech, 2007). Recent studies showed that the micronucleus genome damage biomarker of the CBMN Cyt assay in lymphocytes was predictive of cancer risk and associated with lung cancer risk in humans (Bonassi et al., 2007; El-Zein et al., 2006). The micronucleus index in the CBMN Cyt assay was also shown to be predictive of cardiovascular mortality (Federici et al., 2008; Murgia et al., 2007).

1.9.3.2 The alkaline Comet assay

The Comet assay, or single-cell gel electrophoresis assay (SCGE), is a rapid and sensitive test for assessment of DNA breakage in individual cells, which is widely used in biomonitoring of exposures in occupational and environmental settings as well as in investigating the effects of dietary antioxidant on DNA damage in humans (Møller et al., 2000; Speit and Hartmann, 2005). In combination with glycosylase treatment, the

alkaline Comet assay is able to detect specific oxidized bases (Collins, 2004). Therefore, the Comet assay is also a useful tool for evaluating oxidative stress in cells.

1.10 Role of Se in genome instability/damage

1.10.1 Se and DNA damage

Limited studies have used DNA damage as a biomarker to monitor the association between Se status and baseline genome instability or the effects of Se supplementation on genome health in humans. In a group of New Zealand individuals who were at high risk of prostate cancer, serum Se levels within the range required for saturation of GPx activity (100 ng/ml) were significantly and inversely associated with overall accumulated DNA damage as determined by the Comet assay (P=0.02) (Karunasinghe et al., 2004). A human study showed that supplementation with 247 μg Se as Se-enriched yeast may result in a reduction of oxidative stress, however, no effect on oxidative damage to DNA by measuring of 8-OHdG was evident (El-Bayoumy et al., 2002).

An animal study on ageing dogs supplemented with either Se yeast or Se-met (3 μg or 6 μg /kg/day) suggested a significant decrease in DNA damage as determined by the Comet assay in prostate cells (P<0.01) and lymphocytes (P=0.003), and a significant increase in epithelial cell apoptosis (P=0.04) in the intervention group compared with controls (Waters et al., 2003). Thirunavukkarasu and Sakthisekaran documented that supplementation of Se (4 mg/kg sodium selenite through drinking water) reduced lymphocyte DNA damage determined by the Comet assay in rats bearing hepatoma (P<0.05) (Thirunavukkarasu and Sakthisekaran, 2003). In contrast, mice fed on Se deficient diet had low GPx activities and activation of genes involved in DNA damage (Rao et al., 2001).

In an *in vitro* toxicity study using human lymphocyte cultures, Biswas et al reported Se salts induced chromosome damage and reduced cell division in human lymphocytes (Biswas et al., 2000). Sodium selenite and sodium selenate are highly mitostatic, at a level of 458 μg/l and 837 μg/l respectively, and can induce chromosomal aberration and reduced cell division at a level of 229 μg/l for selenite and, 418 μg/l for selenate or above. However, these levels are at the higher end or beyond the normal physiological range of Se (79 - 395 μg/l) (Smith et al., 2004).

1.10.2 Assumptions and evidence

Se in the correct dose and form may improve genome stability and hence be chemopreventive against cancers. However, the molecular mechanisms of Se compounds' action on genome stability remain largely unclear. The underlying mechanisms may involve reducing DNA damage, stimulation of DNA repair, mediating apoptosis in transformed cells and alteration of expression of genes involved in antioxidative response and genome maintenance.

Reduction of DNA damage

1) Antioxidant activity to reduce oxidative stress

Elevated levels and activity of GPx, a beneficial scavenger of DNA-damaging oxygen free radicals, with Se supplementation may partly and indirectly support the protective role of Se on genome stability (Neve et al., 1988; Thomson et al., 1993). In an isolated system, Roussyn et al showed that 0.1 mM Se-met could suppress peroxynitrite induced single strand break formation by 75% (Roussyn et al., 1996). Rafferty et al reported that Se-met (200 nM) and sodium selenite (50 nM) were protective against oxidative damage to DNA caused by broadband ultraviolet radiation in human keratinocytes, as determined by Fpg-sensitive sites (an indicator of 8-hydroxy-2-deoxygyuanosine photoproduct formation (8OhdG)) (Rafferty et al., 2003).

DNA damage was greatly reduced by preincubation of human keratinocytes with Se-met or selenite. An intervention study in healthy adults (247 μg /day from high Se yeast) suggested that there was a 32% increase in blood glutathione (GSH) levels and a 26% decrease in protein-bound GSH after 9 months in the treatment group (El-Bayoumy et al., 2002). This may indicate a decrease in oxidative stress. However, the levels of 8-OHdG in urine, an indicator of oxidative damage to DNA, were unchanged by Se supplementation.

Involvement in the antioxidant defence system as GPx may not explain the anti-cancer properties of the element at a supra-nutritional level. Moreover, a pharmacologic dose of sodium selenite (126.6 μM) increased 8-OHdG concentrations and induced cytotoxicity in human keratinocytes, whereas Se-met did not (Shen et al., 2001). This result suggested that high Se status/intake effect on oxidative damage to DNA may depend on the chemical form of Se.

2) Telomere shortening

Hepatocytes L-02 cultured in normal medium with addition of sodium selenite at a concentration of both 39.5 μg /l and 197 μg/l significantly increased cellular telomerase activity and human telomerase reverse transcriptase (hTERT) gene expression, compared with those cultured in normal medium (Liu et al., 2003). It has been shown that increased oxidative stress can accelerate telomere-shortening to an extent that induces genome instability, cell senescence and death (Kawanishi and Oikawa, 2004). Selenite at nutritional doses could maintain genome stability of cells through increased telomerase activity and prevention of telomere shortening. However, this hypothesis needs to be tested.

3) Inhibition of DNA adduct formation induced by carcinogens

Selenate inhibited DNA alkylation and reduced DNA adduct formation in an isolated plasmid system (Hamilton and Wilker, 2004). It has been proposed that anionic oxo species such as selenate may behave as nucleophilic targets for the electrophilic alkylating agent and thereby protect DNA damage from carcinogens. However, the required protective concentration of selenate is not achievable *in vivo* (24.6 mM or 246 mM) (Hamilton and Wilker, 2004).

Rats fed on a diet supplemented with selenite or selenate (0.1 or 0.2 mg/kg diet) had significantly fewer 3,2'-dimethyl-4-aminobiphenyl (DMABP)-DNA adducts in the colon ($P<0.05$) than Se deficient rats (Davis et al., 1999). Alteration in activity of cytochrome P450 and glutathione transferase may be involved in this detoxification effect. However, no such effect was observed in the rats fed with Se-met.

Alteration of DNA repair

Se-met indirectly switched on a DNA repair subpathway controlled by the regulatory protein p53 in the human lung cancer cell line H1299 (p53-null), which was transiently transfected with pCMV-p53 encoding wild-type human p53. The authors interpreted this as indicating that the concentration of Se-met (20 μM) was a determinant of basal p53 activity, probably through redox regulation, which is critical to DNA repair (Smith et al., 2004). In another study, Se-met (10 or 20 μM) was shown to enhance the expression of gene *ATR* and *CHK2* in thyroid epithelial cells (Kennedy et al., 2004).

The expression levels of these two genes were used as sensors of DNA damage since both of them are important components of the DNA damage response pathway.

Nasr et al (Nasr et al., 2004) reported that in human MCF-7 (breast cancer cell line) GPx1 transfectant, a significant increase in *Gadd*45 levels (a DNA damage response protein) was observed in response to GPx1 overexpression. However, *Gadd*45 levels did not respond to doubled GPx1 activity after Se supplementation in culture medium in cells with normal GPx1 expression. This finding suggests that Se may enhance DNA damage prevention through GPx1 regulation.

Reducible Se compounds have been shown to release zinc from metallothionein (MT) in isolated systems, which may be available for essential reactions, such as redox control and signalling (Jacob et al., 1998). It is known that zinc finger structures are among the most abundant protein motifs in the eukaryotic genome, which are present in transcription factors and other factors involved in DNA damage signalling and repair, such as poly(ADP-ribose) polymerase-1, formanidopyrimidine-DNA glycosylase (Fpg) and xeroderma pigmentosum group A protein (XPA) (Blessing et al., 2004). However, reducible Se compounds may also inactivate DNA repair by the oxidation of zinc finger structures in DNA repair proteins (Blessing et al., 2004). Under certain conditions, possibly involving Se imbalance, GPx can mimic the effect of hydrogen peroxide to oxidize the zinc finger peptide fragment of transcription factor, SP1 (Giles et al., 2003a; Giles et al., 2003b). It has been shown that selenomethionine at high concentrations (>50 μM) reduces gene repair through a p53-associated pathway correlated with altered cell cycle progression in human colon cancer cells (Schwartz and Kmiec, 2007).

Mediating cell apoptosis

Apoptosis is a critical cellular event that eliminates cells with extensive genome damage and potentially cancerous cells. Usually, at higher concentrations (3 μM to 100 μM) Se inhibits growth in normal and some tumour cells (Sinha and El-Bayoumy, 2004). The cell apoptosis mechanisms involve many molecular targets and various pathways depending on the form of Se and concentration. Some examples are given below.

An *in vitro* study suggested that selenite (500 μM) induced apoptosis in cells, which was probably mediated by activating DNA damage signals, and involved the DNA damage regulator ATM/ATR and Top II-DNA cleavage complex (Zhou et al., 2003).

The natural Se metabolite selenodiglutathione (SDG) induced cell apoptosis more effectively in oral carcinoma cells than in normal oral mucosa cells (Fleming et al., 2001). This may be explained by the SDG mediated induction of the Fas ligand in carcinoma cells while both cell lines express Fas-L receptor constitutively. Fas ligands bind and activate their receptors and then the complex activates caspases to initiate cell apoptosis.

Se-methylselenocysteine has also been shown to induce SKOV-3 ovarian cancer cell apoptosis *in vitro*, which was associated with caspase-3 activation, cleavage of poly(ADP-ribose) polymerase and phospholipase C-gamma1 protein (Yeo et al., 2002).

Alteration of gene expression

It has been suggested that suboptimal Se intake (in the form of Se-met) may induce alterations in gene expression patterns in rats. Low Se status results in a differential gene expression pattern indicative of activation of genes involved in DNA damage, genetic instability, oxidative stress, cell cycle control and a decrease in the expression of genes involved in detoxification (Rao et al., 2001). This study suggests a link between genetic instability and oxidative stress at the transcriptional level, resulting from suboptimal intake of Se.

Both *in vitro* and *in vivo* Se deficiency is associated with DNA hypomethylation. Rats fed with Se-deficient diet had significantly ($P<0.0001$) higher hypomethylated liver and colon DNA than those supplemented with Se as either selenite or Se-met (Davis et al., 2000). DNA isolated from Caco-2 cells (human colon cancer cells) which were not treated with selenite were significantly ($P<0.0001$) hypomethylated compared with cells treated with selenite (1 μmol/l) (Davis et al., 2000). This study does not support the proposal that the biomethylation of selenite competes with cytosine DNA methyltransferase for the methyl donor, S-adenosylmethionine (SAM), and causes hypomethylation. However, possible alternative mechanism was not discussed by the authors. For example, it was also reported that DNA cytosine-5-methyltransferase

(Mtase) activity and growth of human colon carcinoma HCT116 cells were inhibited by p-XSC, a synthetic Se compound (Fiala et al., 1998).

1.11 Conclusion and knowledge gaps

A nutritionally adequate Se intake is important for normal body functioning and a supra-nutritional intake of Se may be required for prevention of certain degenerative diseases including cancer. Genome damage has recently been demonstrated to be the most advanced and reliable measure to determine optimal intakes of micronutrients (Fenech, 2003). Some vulnerable populations may not have adequate Se intake/status to minimise the risk of genome instability and reduce the risk of certain degenerative diseases caused by DNA damage. But the impact of organic Se on genome stability has not been adequately explored. A novel strategy for increasing the intake of Se through the consumption of biofortified high-Se wheat has been proposed (Lyons et al., 2004b). Biofortified high-Se wheat produced in South Australia is potentially a good dietary source of Se for individuals with sub-optimal Se intake. However, the genome health effect of Se supplementation and the optimal dose for minimising genome damage are yet to be determined. The bioavailability and bioefficacy of Se in the form of biofortified high-Se wheat has not been investigated in older men in Australia despite an interest to improve Se status to reduce prostate cancer risk. The impact of high-Se wheat on DNA damage measurement is unknown. The upper limit of Se intake has not been clearly defined in terms of toxicity and DNA damage. These questions can only be answered by a well designed double-blind, randomized, placebo-controlled trial.

Note: Literature considered in this chapter was up to the date of its completion in 2008.

1.12 References

ALLAN, C., LACOURCIERE, G. & STADTMAN, T. (1999) Responsiveness of selenoproteins to dietary selenium. *Annual Review of Nutrition,* 19**,** 1-16.

ALLANDER, E. (1994) Kashin-Beck disease. An analysis of research and public health activities based on a bibliography 1849-1992. *Scandinavian Journal of Rheumatology. Supplement,* 99**,** 1-36.

ALLMANG, C. & KROL, A. (2006) Selenoprotein synthesis: UGA does not end the story. *Biochimie,* 88**,** 1561-1571.

ARO, A., ALFTHAN, G. & VARO, P. (1995) Effects of supplementation of fertilizers on human selenium status in Finland. *Analyst,* 120**,** 841-843.

ARTEEL, G. E., MOSTERT, V., OUBRAHIM, H., BRIVIBA, K., ABEL, J. & SIES, H. (1998) Protection by selenoprotein P in human plasma against peroxynitrite-mediated oxidation and nitration. *Biological Chemistry,* 379**,** 1201-1205.

ARTHUR, J. R. (1999) Functional indicators of iodine and selenium status. *Proceeding*

of the Nutrition Society, 58, 507-12.
ARTHUR, J. (2000) The glutathione peroxidases. *CMLS Cellular and Molecular Life Sciences,* 57**,** 1825-1835.
ARTHUR, J., MCKENZIE, R. & BECKETT, G. (2003) Selenium in the immune system. *Journal of Nutrition,* 133**,** 1457S-1459S.
BARRETT, J., PATTERSON, C., REILLY, C. & TINGGI, U. (1989) *Selenium in the diet of children with phenylketonuria,* London, Royal Society of Chemistry.
BECK, M. A. (1997) Rapid genomic evolution of a non-virulent coxsackievirus B3 in selenium-deficient mice. *Biomedical and Environmental Sciences,* 10**,** 307-315.
BECK, M. A., LEVANDER, O. A. & HANDY, J. (2003) Selenium deficiency and viral infection. *Journal of Nutrition,* 133**,** 1463S-1467S.
BECKETT, G. J. & ARTHUR, J. R. (2005) Selenium and endocrine systems. *Journal of Endocrinology*, 184, 455-65.
BEHN, D., WEISS-NOWAK, C., KALCKLOSCH, M., WESTPHAL, C., GESSNER, H. & KYRIAKOPOULOS, A. (1995) Studies on the distribution and characteristics of new mammalian selenium-containing proteins. *Analyst,* 120**,** 823-825.
BEHNE, D. & KYRIAKOPOULOS, A. (2001) Mammalian selenium-containing proteins. *Annual Review of Nutrition,* 21**,** 453-473.
BIRRINGER, M., PILAWA, S. & FLOHE, L. (2002) Trends in selenium biochemistry. *Natural Product Reports*, 19**,** 693-718.
BISWAS, S., TALUKDER, G. & SHARMA, A. (2000) Chromosome damage induced by selenium salts in human peripheral lymphocytes. *Toxicol In Vitro,* 14**,** 405-408.
BLESSING, H., KRAUS, S., HEINDL, P., BAL, W. & HARTWIG, A. (2004) Interaction of selenium compounds with zinc finger proteins involved in DNA repair. *European Journal of Biochemistry,* 271**,** 3190-3199.
BONASSI, S., HAGMAR, L., STROMBERG, U., MONTAGUD, A. H., TINNERBERG, H., FORNI, A., HEIKKILA, P., WANDERS, S., WILHARDT, P., HANSTEEN, I. L., KNUDSEN, L. E. & NORPPA, H. (2000) Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health. *Cancer Research,* 60, 1619-25.
BONASSI, S., ZNAOR, A., CEPPI, M., LANDO, C., CHANG, W. P., HOLLAND, N., KIRSCH-VOLDERS, M., ZEIGER, E., BAN, S., BARALE, R., BIGATTI, M. P., BOLOGNESI, C., CEBULSKA-WASILEWSKA, A., FABIANOVA, E., FUCIC, A., HAGMAR, L., JOKSIC, G., MARTELLI, A., MIGLIORE, L., MIRKOVA, E., SCARFI, M. R., ZIJNO, A., NORPPA, H. & FENECH, M. (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis,* 28**,** 625-631.
BRIGELIUS-FLOHE, R., BANNING, A. & SCHNURR, K. (2003) Selenium-dependent enzymes in endothelial cell function. *Antioxidants and Redox Signalling,* 5**,** 205-215.
BRIGELIUS-FLOHE, R., MULLER, C., MENARD, J., FLORIAN, S., SCHMEHL, K. & WINGLER, K. (2001) Functions of GI-GPx: lessons from selenium-dependent expression and intracellular localization. *Biofactors,* 14**,** 101-106.
BROOME, C. S., MCARDLE, F., KYLE, J. A., ANDREWS, F., LOWE, N. M., HART, C. A., ARTHUR, J. R. & JACKSON, M. J. (2004) An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status. *American Journal of Clinical Nutrition,* 80**,** 154-162.

BROUWER, I. A., VAN DUSSELDORP, M. & WEST, C. E. Steegers-Theunissen, R. P. (2001) Bioavailability and bioefficacy of folate and folic acid in man. *Nutrition Research Reviews,* 14, 267-94.

BROWN, K. & ARTHUR, J. (2001) Selenium, selenoproeins and human health: a review. *Public Health Nutrition,* 4**,** 593-599.

BURK, R. (2002) Selenium, an antioxidant nutrient. *Nutrition in Clinical Care,* 5**,** 75-79.

BURK, R., NORSWORTHY, B., HILL, K., MOTLEY, A. & BYRNE, D. (2006) Effects of chemical form of selenium on plasma biomarkers in a high-dose human supplementation trial. *Cancer Epidemiology, Biomarkers and Prevention,* 15**,** 804-810.

CAO, Y. Z., MADDOX, J. F., MASTRO, A. M., SCHOLZ, R. W., HILDENBRANDT, G. & REDDY, C. C. (1992) Selenium deficiency alters the lipoxygenase pathway and mitogenic response in bovine lymphocytes. *Journal of Nutrition,* 122**,** 2121-2127.

CHO, S. D., JIANG, C., MALEWICZ, B., DONG, Y., YOUNG, C. Y., KANG, K. S., LEE, Y. S., IP, C. & LU, J. (2004) Methyl selenium metabolites decrease prostate-specific antigen expression by inducing protein degradation and suppressing androgen-stimulated transcription. *Molecular Cancer Therapeutics,* 3**,** 605-611.

CLARK, L., DALKIN, B., KRONGRAD, A., COMBS, G., JR., TURNBULL, B., SLATE, E., WITHERINGTON, R., HERLONG, J., JANOSKO, E., CARPENTER, D., BOROSSO, C., FALK, S. & ROUNDER, J. (1998) Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *British Journal of Urology,* 81**,** 730-734.

COLLINS, A. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology,* 26**,** 249-261.

COMBS, G., JR. & GRAY, W. (1998) Chemopreventive agents: selenium. *Pharmacology and Therapeutics,* 79**,** 179-192.

COMBS, G. F., JR. (2001a) Considering the mechanisms of cancer prevention by selenium. *Advances in Experimental Medicine and Biology,* 492**,** 107-117.

COMBS, G. F., JR. (2001b) Selenium in global food systems. *British Journal of Nutrition,* 85**,** 517-547.

COMBS, G. F., JR., CLARK, L. C. & TURNBULL, B. W. (2001) An analysis of cancer prevention by selenium. *Biofactors,* 14**,** 153-159.

COURTEMANCHE, C., HUANG, A. C., ELSON-SCHWAB, I., KERRY, N., NG, B. Y. & AMES, B. N. (2004) Folate deficiency and ionizing radiation cause DNA breaks in primary human lymphocytes: a comparison. *FASEB Journal,* 18**,** 209-211.

DANIELS, L. (1996) Selenium metabolism and bioavailability. *Biological Trace Element Research,* 54**,** 185-199.

DAVIS, C., FENG, Y., HEIN, D. & FINLEY, J. (1999) The chemical form of selenium influences 3,2'-dimethyl-4-aminobiphenyl-DNA adduct formation in rat colon. *Journal of Nutrition,* 129**,** 63-69.

DAVIS, C., UTHUS, E. & FINLEY, J. (2000) Dietary selenium and arsenic affect DNA methylation in vitro in Caco-2 cells and in vivo in rat liver and colon. *Journal of Nutrition,* 130**,** 2903-2909.

DE JONG, N., GIBSON, R., THOMSON, C., FERGUSON, E., MCKENZIE, J., GREEN, T. & HORWATH, C. (2001) Selenium and zinc status are suboptimal in a sample of older New Zealand women in a community-based study. *Journal of Nutrition,* 131**,** 2677-2684.

DIAMOND, A. M., CHOI, I. S., CRAIN, P. F., HASHIZUME, T., POMERANTZ, S. C., CRUZ, R., STEER, C. J., HILL, K. E., BURK, R. F., MCCLOSKEY, J. A. & ET AL. (1993) Dietary selenium affects methylation of the wobble nucleoside in the anticodon of selenocysteine tRNA([Ser]Sec). *Journal of Biological Chemistry,* 268**,** 14215-14223.

DIAMOND, T. L., KIMATA, J. & KIM, B. (2001) Identification of a simian immunodeficiency virus reverse transcriptase variant with enhanced replicational fidelity in the late stage of viral infection. *Journal of Biological Chemistry,* 276**,** 23624-23631.

DIPLOCK, A. T. (1981) Metabolic and functional defects in selenium deficiency. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences,* 294**,** 105-117.

DREHER, I., SCHMUTZLER, C., JAKOB, F. & KOHRLE, J. (1997) Expression of selenoproteins in various rat and human tissues and cell lines. *Journal of Trace Elements in Medicine and Biology,* 11**,** 83-91.

DUFFIELD-LILLICO, A., REID, M., TURNBULL, B., COMBS, G., JR., SLATE, E., FISCHBACH, L., MARSHALL, J. & CLARK, L. (2002) Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 630-639.

DUFFIELD-LILLICO, A. J., DALKIN, B. L., REID, M. E., TURNBULL, B. W., SLATE, E. H., JACOBS, E. T., MARSHALL, J. R. & CLARK, L. C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU International,* 91**,** 608-612.

DUFFIELD, A., THOMSON, C., HILL, K. & WILLIAMS, S. (1999) An estimation of selenium requirements for New Zealanders. *American Journal of Clinical Nutrition,* 70**,** 896-903.

DUMONT, E., OGRA, Y., VANHAECKE, F., SUZUKI, K. T. & CORNELIS, R. (2006) Liquid chromatography-mass spectrometry (LC-MS): a powerful combination for selenium speciation in garlic (Allium sativum). *Analytical and Bioanalytical Chemistry,* 384**,** 1196-1206.

EL-BAYOUMY, K. (2001) The protective role of selenium on genetic damage and on cancer. *Mutation Research,* 475**,** 123-139.

EL-BAYOUMY, K., RICHIE JR, J., BOYIRI, T., KOMNINOU, D., PROKOPCZK, B., TRUSHIN, N., KLEINMAN, W., COX, J., PITTMAN, B. & COLOSIMO, S. (2002) Influence of selenium-enriched yeast supplementation on biomarkers of oxidative damage and hormone status in helathy adult makes: a clinical pilot study. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 1459-1465.

EL-BAYOUMY, K. & SINHA, R. (2005) Molecular chemoprevention by selenium: a genomic approach. *Mutation Research,* 591**,** 224-236.

ELSOM, R., SANDERSON, P., HESKETH, J. E., JACKSON, M. J.,FAIRWEATHER-TAIT, S. J., AKESSON, B., HANDY, J. & ARTHUR, J. R. (2006) Functional markers of selenium status: UK Food Standards Agency workshop report. *British Journal of Nutrition,* 96, 980-4.

EL-ZEIN, R. A., SCHABATH, M. B., ETZEL, C. J., LOPEZ, M. S., FRANKLIN, J. D. & SPITZ, M. R. (2006) Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Research,* 66**,** 6449-6456.

FAIRWEATHER-TAIT, S. J. (1997a) Bioavailability of selenium. *European Journal Clinical Nutrition,* 51 Suppl 1, S20-3

FAIRWEATHER-TAIT, S. J. (1997b) From absorption and excretion of minerals ... to the importance of bioavailability and adaptation. *British Journal of Nutrition*, 78 Suppl 2, S95-100.

FEDERICI, C., BOTTO, N., MANFREDI, S., RIZZA, A., DEL FIANDRA, M. & ANDREASSI, M. G. (2008) Relation of increased chromosomal damage to future adverse cardiac events in patients with known coronary artery disease. *American Journal of Cardiology,* 102**,** 1296-1300.

FENECH, M. (2001) The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutation Research,* 475**,** 57-67.

FENECH, M. (2003) Nutritional treatment of genome instability: a paradigm shift in disease prevention and in the setting of recommended dietary allowances. *Nutrition Research Reviews,* 16**,** 109-122.

FENECH, M. (2006) Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research,* 600**,** 58-66.

FENECH, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nature Protocol,* 2**,** 1084-1104.

FENECH, M. & FERGUSON, L. (2001) Vitamins/minerals and genomic stability in humans. *Mutation Research,* 475**,** 1-6.

FIALA, E., STARETZ, M., PANDYA, G., EL-BAYOUMY, K. & HAMILTON, S. (1998) Inhibition of DNA cytosine methyltransferase by chemopreventive selenium compounds, determined by an improved assay for DNA cytosine methyltransferase and DNA cytosine methylation. *Carcinogenesis,* 19**,** 597-604.

FINLEY, J. W. (2005) Selenium accumulation in plant foods. *Nutrition Reviews,* 63**,** 196-202.

FINLEY, J. W. (2006) Bioavailability of selenium from foods. *Nutrition Reviews,* 64**,** 146-151.

FINLEY, J. W., GRUSAK, M. A., KECK, A. S. & GREGOIRE, B. R. (2004) Bioavailability of selenium from meat and broccoli as determined by retention and distribution of 75Se. *Biological Trace Element Research,* 99**,** 191-209.

FLEMING, J., GHOSE, A. & HARRISON, P. (2001) Molecular mechanisms of cancer prevention by selenium compounds. *Nutrition and Cancer,* 40**,** 42-49.

FLOHÉ, L., GUNZLER, W. & SCHOCK, H. (1973) Glutathione perosidase: a selenoenzyme. *FEBS Letters,* 32**,** 132.

FLORES-MATEO, G., NAVAS-ACIEN, A., PASTOR-BARRIUSO, R. & GUALLAR, E. (2006) Selenium and coronary heart disease: a meta-analysis. *American Journal of Clinical Nutrition,* 84**,** 762-773.

FOOD AND NUTRITION BOARD (2000) *Dietary reference intakes for vitamin C, vitamin E, selenium and carotenoids,* Washington DC, National Academy Press.

FORDYCE, F. (2005) Selenium deficiency and toxicity in the environment. IN SELINUS, O., ALLOWAY, B., CENTENO, J., FINKELMAN, R., FUGE, R., LINDH, U. & SMEDLEY, P. (Eds.) *Essentials of Medical Geology.* London, Elsevier.

FOSTER, S. J., KRAUS, R. J. & GANTHER, H. E. (1986a) Formation of dimethyl selenide and trimethylselenonium from selenobetaine in the rat. *Archives of Biochemistry and Biophysics,* 247**,** 12-19.

FOSTER, S. J., KRAUS, R. J. & GANTHER, H. E. (1986b) The metabolism of selenomethionine, Se-methylselenocysteine, their selenonium derivatives, and trimethylselenonium in the rat. *Archives of Biochemistry and Biophysics,* 251**,** 77-86.

FRIEDBERG, E. (2003) DNA damage and repair. *Nature,* 421**,** 436-440.

FUTREAL, P., LIU, Q., SHATTUCK-EIDENS, D., COCHRAN, C., HARSHMAN, K., TAVTIGIAN, S., BENNETT, L., HAUGEN-STRANO, A., SWENSEN, J. & MIKI, Y. (1994) BRCA1 mutations in primary breast and ovarian carcinomas. *Science,* 266**,** 120-122.

GANTHER, H. (1999) Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. *Carcinogenesis,* 20**,** 1657-1666.

GILES, N. M., GILES, G. I., HOLLEY, J. E., GUTOWSKI, N. J. & JACOB, C. (2003a) Targeting oxidative stress-related diseases: organochalcogen catalysts as redox sensitizers. *Biochemical Pharmacology,* 66**,** 2021-2028.

GILES, N. M., GUTOWSKI, N. J., GILES, G. I. & JACOB, C. (2003b) Redox catalysts as sensitisers towards oxidative stress. *FEBS Letters,* 535**,** 179-182.

GOLUBKINA, N. A. & ALFTHAN, G. (2002) Selenium status of pregnant women and newborns in the former Soviet Union. *Biological Trace Element Research,* 89**,** 13-23.

GOODMAN, G. E., SCHAFFER, S., BANKSON, D. D., HUGHES, M. P. & OMENN, G. S. (2001) Predictors of serum selenium in cigarette smokers and the lack of association with lung and prostate cancer risk. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 1069-1076.

GOPALAKRISHNA, R., CHEN, Z. H. & GUNDIMEDA, U. (1997) Selenocompounds induce a redox modulation of protein kinase C in the cell, compartmentally independent from cytosolic glutathione: its role in inhibition of tumor promotion. *Archives of Biochemistry and Biophysics,* 348**,** 37-48.

GRADY, W. M. (2004) Genomic instability and colon cancer. *Cancer and Metastasis Reviews,* 23**,** 11-27.

GRAHAM, R., WELCH, R. & BOUIS, H. (2001) Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. *Advances in Agronomy,* 70**,** 77-142.

GRIFFITHS, J. C., MATULKA, R. A. & POWER, R. (2006) Acute and subchronic toxicity studies on Sel-Plex, a standardized, registered high-selenium yeast. *International Journal of Toxicology,* 25**,** 465-476.

GROMER, S., EUBEL, J. K., LEE, B. L. & JACOB, J. (2005) Human selenoproteins at a glance. *Cellular and Molecular Life Sciences,* 62**,** 2414-2437.

GROMER, S., URIG, S. & BECKER, K. (2004) The thioredoxin system--from science to clinic. *Medicinal Research Reviews,* 24**,** 40-89.

GU, Q. P., SUN, Y., REAM, L. W. & WHANGER, P. D. (2000) Selenoprotein W accumulates primarily in primate skeletal muscle, heart, brain and tongue. *Molecular Cell Biochemistry,* 204, 49-56.

GUIMARAES, M. J., PETERSON, D., VICARI, A., COCKS, B. G., COPELAND, N. G., GILBERT, D. J., JENKINS, N. A., FERRICK, D. A., KASTELEIN, R. A., BAZAN, J. F. & ZLOTNIK, A. (1996) Identification of a novel selD homolog from eukaryotes, bacteria, and archaea: is there an autoregulatory mechanism in selenocysteine metabolism? *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 15086-15091.

GUPTA, U. & WINTER, K. (1975) Selenium content of soils and crops and the effects of lime and sulfur on plant selenium. *Canadian Journal of Soil Science,* 55**,** 161-166.

HAMBIDGE, M. (2003) Biomarkers of trace mineral intake and status. *Journal of Nutrition,* 133**,** 948S-955S.

HAMILTON, E. & WILKER, J. (2004) Inhibition of DNA alkylation damage with inorganic salts. *Journal of Biological Inorganic Chemistry,* 9**,** 894-902.

HARTMAN, T. J., TAYLOR, P. R., ALFTHAN, G., FAGERSTROM, R., VIRTAMO, J., MARK, S. D., VIRTANEN, M., BARRETT, M. J. & ALBANES, D. (2002) Toenail selenium concentration and lung cancer in male smokers (Finland). *Cancer Causes and Control,* 13**,** 923-928.

HARTWIG, A., ASMUSS, M., BLESSING, H., HOFFMANN, S., JAHNKE, G., KHANDELWAL, S., PELZER, A. & BURKLE, A. (2002) Interference by toxic metal ions with zinc-dependent proteins involved in maintaining genomic stability. *Food and Chemical Toxicology,* 40**,** 1179-1184.

HATFIELD, D. L. & GLADYSHEV, V. N. (2002) How selenium has altered our understanding of the genetic code. *Molecular and Cellular Biology*, 22**,** 3565-3576.

HATHCOCK, J. (2004) *Vitamin and Mineral Safety,* Washington DC, Council for Responsible Nutrition.

HAWKES, W. C., KELLEY, D. S. & TAYLOR, P. C. (2001) The effects of dietary selenium on the immune system in healthy men. *Biological Trace Element Research,* 81**,** 189-213.

HELMERSSON, J., ARNLOV, J., VESSBY, B., LARSSON, A., ALFTHAN, G. & BASU, S. (2005) Serum selenium predicts levels of F2-isoprostanes and prostaglandin F2alpha in a 27 year follow-up study of Swedish men. *Free Radical Research,* 39**,** 763-770.

HILL, K. E., XIA, Y., AKESSON, B., BOEGLIN, M. E. & BURK, R. F. (1996) Selenoprotein P concentration in plasma is an index of selenium status in selenium-deficient and selenium-supplemented Chinese subjects. *Journal of Nutrition,* 126**,** 138-145.

HOEIJMAKERS, J. H. (2001) Genome maintenance mechanisms for preventing cancer. *Nature,* 411**,** 366-374.

IP, C., DONG, Y. & GANTHER, H. E. (2002) New concepts in selenium chemoprevention. *Cancer and Metastasis Reviews,* 21**,** 281-289.

IP, C., HAYES, C., BUDNICK, R. M. & GANTHER, H. E. (1991) Chemical form of selenium, critical metabolites, and cancer prevention. *Cancer Research,* 51**,** 595-600.

ITO, Y., KAJKENOVA, O., FEUERS, R. J., UDUPA, K. B., DESAI, V. G., EPSTEIN, J., HART, R. W. & LIPSCHITZ, D. A. (1998) Impaired glutathione peroxidase activity accounts for the age-related accumulation of hydrogen peroxide in activated human neutrophils. *Journals of Gerontology. Series A, Biological Sciences and Medical Sciences,* 53**,** M169-175.

JACKSON, M. J., BROOME, C. S. & MCARDLE, F. (2003) Marginal dietary selenium intakes in the UK: are there functional consequences? *Journal of Nutrition*, 133 5 Suppl 1, 1557S-9S.

JACOB, C., MARET, W. & VALLEE, B. (1998) Ebselen, a selenium containing redox drug, release zinc from metallothionein. *Biochemical and Biophysical Research Communications,* 248**,** 569-573.

KAJIYAMA, H., MURASE, K., MIYAZAKI, T., ISOMOTO, H., FUKUDA, Y., YAMAZAWA, N., SODA, H., TAKESHIMA, F., MIZUTA, Y., MURATA, I. & KOHNO, S. (2001) Micronutrient status and glutathione peroxidase in bedridden patients on tube feeding. *Journal of International Medical Research,* 29**,** 181-188.

KARDINAAL, A. F., KOK, F. J., KOHLMEIER, L., MARTIN-MORENO, J. M., RINGSTAD, J., GOMEZ-ARACENA, J., MAZAEV, V. P., THAMM, M., MARTIN, B. C., ARO, A., KARK, J. D., DELGADO-RODRIGUEZ, M., RIEMERSMA, R. A., VAN 'T VEER, P. & HUTTUNEN, J. K. (1997) Association between toenail selenium and risk of acute myocardial infarction in

European men. The EURAMIC Study. European Antioxidant Myocardial Infarction and Breast Cancer. *American Journal of Epidemiology,* 145**,** 373-379.

KARUNASINGHE, N., RYAN, J., TUCKEY, J., MASTERS, J., JAMIESON, M., CLARKE, L., MARSHALL, J. & FERGUSON, L. (2004) DNA stability and serum selenium levels in a high-risk group for prostate cancer. *Cancer Epidemiology, Biomarkers and Prevention,* 13**,** 391-397.

KAWANISHI, S. & OIKAWA, S. (2004) Mechanism of telomere shortening by oxidative stress. *Annals of the New York Academy of Sciences,* 1019**,** 278-284.

KENNEDY, A., WARE, J., GUAN, J., DONAHUE, J., BIAGLOW, J., ZHOU, Z., STEWART, J., VAZQUEZ, M. & WAN, X. (2004) Selenomethionine protects against adverse biological effects induced by space radiation. *Free Radical Biology and Medicine,* 36**,** 259-266.

KIREMIDJIAN-SCHUMACHER, L. & ROY, M. (1998) Selenium and immune function. *Zeitschrift fur Ernahrungswissenschaft,* 37 Suppl 1**,** 50-56.

KIREMIDJIAN-SCHUMACHER, L., ROY, M., WISHE, H., COHEN, M. & STOTZKY, G. (1994) Supplementation with selenium and human immune cell functions. II. Effect on cytotoxic lymphocytes and natural killer cells. *Biological Trace Element Research,* 41**,** 115-127.

KIREMIDJIAN-SCHUMACHER, L. & STOTZKY, G. (1987) Selenium and immune responses. *Environmental Research,* 42**,** 277-303.

KOBAYASHI, Y., OGRA, Y., ISHIWATA, K., TAKAYAMA, H., AIMI, N. & SUZUKI, K. T. (2002) Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 15932-15936.

KOHRL, J., BRIGELIUS-FLOHE, R., BOCK, A., GARTNER, R., MEYER, O. & FLOHE, L. (2000) Selenium in biology: facts and medical perspectives. *Biological Chemistry,* 381**,** 849-864.

KOHRLE, J. (2005) Selenium and the control of thyroid hormone metabolism. *Thyroid,* 15, 841-53.

KOROTKOV, K. V., NOVOSELOV, S. V., HATFIELD, D. L. & GLADYSHEV, V. N. (2002) Mammalian selenoprotein in which selenocysteine (Sec) incorporation is supported by a new form of Sec insertion sequence element. *Molecular and Cellular Biology,* 22**,** 1402-1411.

KRYUKOV, G. V., CASTELLANO, S., NOVOSELOV, S. V., LOBANOV, A. V., ZEHTAB, O., GUIGO, R. & GLADYSHEV, V. N. (2003) Characterization of mammalian selenoproteomes. *Science,* 300**,** 1439-1443.

KUEHNELT, D., KIENZL, N., TRAAR, P., LE, N. H., FRANCESCONI, K. A. & OCHI, T. (2005) Selenium metabolites in human urine after ingestion of selenite, L-selenomethionine, or DL-selenomethionine: a quantitative case study by HPLC/ICPMS. *Analytical and Bioanalytical Chemistry,* 383**,** 235-246.

KUROKI, F., MATSUMOTO, T. & IIDA, M. (2003) Selenium is depleted in Crohn's disease on enteral nutrition. *Digestive Diseases,* 21**,** 266-270.

LEE, B. J., WORLAND, P. J., DAVIS, J. N., STADTMAN, T. C. & HATFIELD, D. L. (1989) Identification of a selenocysteyl-tRNA(Ser) in mammalian cells that recognizes the nonsense codon, UGA. *Journal of Biological Chemistry,* 264**,** 9724-9727.

LEE, O., MOON, J. & CHUNG, Y. (2003) The relationship between serum selenium levels and lipid profiles in adult women. *Journal of Nutritional Science and Vitaminology,* 49**,** 397-404.

LEI, X. G., CHENG, W. H. & MCCLUNG, J. P. (2007) Metabolic regulation and function of glutathione peroxidase-1. *Annual Review of Nutrition,* 27, 41-61.

LEVANDER, O., ALFTHAN, G., ARVILOMMI, H., GREF, C., HUTTUNEN, J., KATAJA, M., KOIVISTOINEN, P. & PIKKARAINEN, J. (1983a) Bioavailability of selenium to Finnish men as assessed by platelet glutathione peroxidase activity and other blood parameters. *American Journal of Clinical Nutrition,* 37**,** 887-897.

LEVANDER, O. & BURK, R. (1996) Selenium. IN ZIEGLER, E. & JR FILER, L. (Eds.) *Present knowledge in nutrition.* 7th ed. Washington, DC, ILSI Press.

LEVANDER, O., DELOACH, D., MORRIS, V. & MOSER, P. (1983b) Platelet glutathione peroxidase activity as an index of selenium status in rats. *Journal of Nutrition,* 113**,** 55-63.

LEVANDER, O. A. & BECK, M. A. (1997) Interacting nutritional and infectious etiologies of Keshan disease. Insights from coxsackie virus B-induced myocarditis in mice deficient in selenium or vitamin E. *Biological Trace Element Research,* 56**,** 5-21.

LEVANDER, O. A. & WHANGER, P. D. (1996) Deliberations and evaluations of the approaches, endpoints and paradigms for selenium and iodine dietary recommendations. *Journal of Nutrition,* 126**,** 2427S-2434S.

LINDAHL, T. & WOOD, R. D. (1999) Quality control by DNA repair. *Science,* 286**,** 1897-1905.

LIU, Q., WANG, H., HU, D., DING, C., XIAO, H., XU, H., SHU, B. & XU, S. (2003) Effects of sodium selenite on telomerase activity and telomere length. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai),* 35**,** 1117-1122.

LU, J. & JIANG, C. (2005) Selenium and cancer chemoprevention: hypotheses integrating the actions of selenoproteins and selenium metabolites in epithelial and non-epithelial target cells. *Antioxidants and Redox Signalling,* 7**,** 1715-1727.

LU, J., JIANG, C., KAECK, M., GANTHER, H., VADHANAVIKIT, S., IP, C. & THOMPSON, H. (1995) Dissociation of the genotoxic and growth inhibitory effects of selenium. *Biochemical Pharmacology,* 50**,** 213-219.

LU, T., PAN, Y., KAO, S.-K., LI, C., KOHANE, I., CHAN, J. & YANKER, B. (2004) Gene regulation and DNA damage in the aging human brain. *Nature,* 42**,** 883-891.

LYONS, G., JUDSON, G., STANGOULIS, J., PALMER, L., JONES, J. & GRAHAM, R. (2004a) Trends in selenium status of South Australians. *Medical Journal of Australia,* 180**,** 383-386.

LYONS, G., LEWIS, J., LORIMER, M., HOLLOWAY, R., BRACE, D., STANGOULIS, J. & GRAHAM, R. (2004b) High-selenium wheat: agronomic biofortification strategies to improve human nutrition. *Food, Agriculture & Environment,* 2**,** 171-178.

LYONS, G., STANGOULIS, J. & GRAHAM, R. (2003a) High-selenium wheat: biofortification for better health. *Nutr Res Rev,* 16**,** 45-60.

LYONS, G., STANGOULIS, J. & GRAHAM, R. (2003b) Nutriprevention of disease with high-selenium wheat. *Journal of the Australian College of Nutritional and Environmental Medicine,* 22**,** 3-9.

LYONS, G. H., JUDSON, G. J., STANGOULIS, J. C., PALMER, L. T., JONES, J. A. & GRAHAM, R. D. (2004c) Trends in selenium status of South Australians. *Med J Aust,* 180**,** 383-386.

MCKENZIE, R. C., ARTHUR, J. R. & BECKETT, G. J. (2002) Selenium and the regulation of cell signaling, growth, and survival: molecular and mechanistic aspects. *Antioxidants and Redox Signalling,* 4**,** 339-351.

MELTZER, H., NORHEIM, G., LOKEN, E. & HOLM, H. (1992) Supplementation with wheat selenium induces a dose-dependent response in serum and urine of a Se-replete population. *British Journal of Nutrition,* 67**,** 287-294.

MERTZ, W. (1998) A perspective on mineral standards. *Journal of Nutrition,* 128**,** 375S-378S.

MØLLER, P., KNUDSEN, L., LOFT, S. & WALLIN, H. (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiology, Biomarkers and Prevention,* 9**,** 1005-1015.

MORENO-REYES, R., SUETENS, C., MATHIEU, F., BEGAUX, F., ZHU, D., RIVERA, M. T., BOELAERT, M., NEVE, J., PERLMUTTER, N. & VANDERPAS, J. (1998) Kashin-Beck osteoarthropathy in rural Tibet in relation to selenium and iodine status. *New England Journal of Medicine,* 339**,** 1112-1120.

MORENO-REYES, R., SUETENS, C., MATHIEU, F., BEGAUX, F., ZHU, D., RIVERA, T., BOELAERT, M., NEVE, J., PERLMUTTER, N. & VANDERPAS, J. (2001) Kashin-Beck disease and iodine deficiency in Tibet. *International Orthopaedics,* 25**,** 164-166.

MULLER, A. S. & PALLAUF, J. (2003) Effect of increasing selenite concentrations, vitamin E supplementation and different fetal calf serum content on GPx1 activity in primary cultured rabbit hepatocytes. *Journal of Trace Elements in Medicine and Biology,* 17**,** 183-192.

MURGIA, E., MAGGINI, V., BARALE, R. & ROSSI, A. M. (2007) Micronuclei, genetic polymorphisms and cardiovascular disease mortality in a nested case-control study in Italy. *Mutation Research,* 621**,** 113-118.

NASR, M., FEDELE, M., ESSER, K. & DIAMOND, A. (2004) GPx-1 modulates Akt and P70S6K phosphorylation and Gadd45 levels in MCF-7 cells. *Free Radical Biology and Medicine,* 37**,** 187-195.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL, N. (2006) *Nutrition reference values for Australia and New Zealand,* Canberra, NHMRC, Wellington: Ministry of Health.

NELSON, H. K., SHI, Q., VAN DAEL, P., SCHIFFRIN, E. J., BLUM, S., BARCLAY, D., LEVANDER, O. A. & BECK, M. A. (2001) Host nutritional selenium status as a driving force for influenza virus mutations. *FASEB Journal,* 15**,** 1846-1848.

NEVE, J. (1991) Methods in determination of selenium states. *Journal of Trace Elements and Electrolytes in Health and Disease,* 5**,** 1-17.

NEVE, J. (1995) Human selenium supplementation as assessed by changes in blood selenium concentration and glutathione peroxidase activity. *Journal of Trace Elements in Medicine and Biology,* 9**,** 65-73.

NEVE, J. (2002) Selenium as a 'nutraceutical': how to conciliate physiological and supra-nutrional effects for an essential trace element. *Current Opinion in Clinical Nutrition and Metabolic Care,* 5**,** 659-663.

NEVE, J., VERTONGEN, F. & CAPEL, P. (1988) Selenium supplementation in healthy Belgian adults: response in platelet glutathione peroxidase activity and other blood indices. *American Journal of Clinical Nutrition,* 48**,** 139-143.

NOMURA, A. M., LEE, J., STEMMERMANN, G. N. & COMBS, G. F., JR. (2000) Serum selenium and subsequent risk of prostate cancer. *Cancer Epidemiology, Biomarkers and Prevention,* 9**,** 883-887.

OKUNO, T., MOTOBAYASHI, S., UENO, H. & NAKAMURO, K. (2005a) Identification of mouse selenomethionine alpha,gamma-elimination enzyme: cystathionine gamma-lyase catalyzes its reaction to generate methylselenol. *Biological Trace Element Research,* 108**,** 245-257.

OKUNO, T., MOTOBAYASHI, S., UENO, H. & NAKAMURO, K. (2005b) Purification and characterization of mouse hepatic enzyme that converts

selenomethionine to methylselenol by its alpha,gamma-elimination. *Biological Trace Element Research,* 106**,** 77-94.

PAGMANTIDIS, V., BERMANO, G., VILLETTE, S., BROOM, I., ARTHUR, J. & HESKETH, J. (2005) Effects of Se-depletion on glutathione peroxidase and selenoprotein W gene expression in the colon. *FEBS Letters***,** 792-796.

PATCH, C., TAPSELL, L. & WILLIAMS, P. (2004) Dietetics and functional foods. *Nutrition and Dietetics,* 61**,** 22-29.

PATCHING, S. G. & GARDINER, P. H. (1999) Recent developments in selenium metabolism and chemical speciation: a review. *Journal of Trace Elements in Medicine and Biology,* 13**,** 193-214.

PETROZZI, L., LUCETTI, C., GAMBACCINI, G., BERNARDINI, S., DEL DOTTO, P., MIGLIORE, L., SCARPATO, R. & BONUCCELLI, U. (2001) Cytogenetic analysis oxidative damage in lymphocytes of Parkinson's disease patients. *Neurological Sciences,* 22**,** 83-84.

PETROZZI, L., LUCETTI, C., SCARPATO, R., GAMBACCINI, G., TRIPPI, F., BERNARDINI, S., DEL DOTTO, P., MIGLIORE, L. & BONUCCELLI, U. (2002) Cytogenetic alterations in lymphocytes of Alzheimer's disease and Parkinson's disease patients. *Neurological Sciences,* 23**,** S97-98.

PRABHU, K. S., ZAMAMIRI-DAVIS, F., STEWART, J. B., THOMPSON, J. T., SORDILLO, L. M. & REDDY, C. C. (2002) Selenium deficiency increases the expression of inducible nitric oxide synthase in RAW 264.7 macrophages: role of nuclear factor-kappaB in up-regulation. *Biochemical Journal,* 366**,** 203-209.

RAFFERTY, T., GREEN, M., LOWE, J., ARLETT, C., HUNTER, J., BECKETT, G. & MCKENZIE, R. (2003) Effects of selenium compounds on induction of DNA damage by broadband ultraviolet radiation in human keratinocytes. *British Journal of Dermatology,* 148**,** 1001-1009.

RAO, L., PUSCHNER, B. & PROLLA, T. (2001) Gene expression profiling of low selenium status in the mouse intestine: transcriptional activation of genes linked to DNA damage, cell cycle control and oxidative stress. *Journal of Nutrition,* 131**,** 3175-3181.

RAYMAN, M. (2000a) The importance of selenium to human health. *Lancet,* 356**,** 233-241.

RAYMAN, M. (2000b) The importance of selenium to human health. *The Lancet,* 356**,** 233-241.

RAYMAN, M. (2004) The use of high-selenium yeast to raise selenium status: how does it measure up? *British Journal of Nutrition,* 92**,** 557-573.

RAYMAN, M. P. (2005) Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society,* 64**,** 527-542.

REID, M. E., DUFFIELD-LILLICO, A. J., GARLAND, L., TURNBULL, B. W., CLARK, L. C. & MARSHALL, J. R. (2002) Selenium supplementation and lung cancer incidence: an update of the nutritional prevention of cancer trial. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 1285-1291.

REILLY, C. (1996) *Selenium in food and health,* London & New York, Blackie Academic & Professional.

REIMUND, J. M., ARONDEL, Y., DUCLOS, B. & BAUMANN, R. (2000) Vitamins and trace elements in home parenteral nutrition patients. *Journal of Nutrition, Health and Aging,* 4**,** 13-18.

ROBBERECHT, H. & DEELSTRA, H. (1994) Factors influencing blood selenium concentration values: a literature review. *Journal of Trace Elements and Electrolytes in Health and Disease,* 8**,** 129-143.

ROUSSYN, I., BRIVIBA, K., MASUMOTO, H. & SIES, H. (1996) Selenium-containing compounds protect DNA from single strand breaks caused by peroxynitrite. *Archives of Biochemistry and Biophysics,* 330**,** 216-218.

ROY, M., KIREMIDJIAN-SCHUMACHER, L., WISHE, H., COHEN, M. & STOTZKY, G. (1994) Supplementation with selenium and human immune cell functions. I. Effect on lymphocyte proliferation and interleukin 2 receptor expression. *Biological Trace Element Research,* 41**,** 103-114.

ROY, M., KIREMIDJIAN-SCHUMACHER, L., WISHE, H., COHEN, M. & STOTZKY, G. (1995) Supplementation with selenium restores age-related decline in immune cell function. *Proceedings of the Society for Experimental Biology and Medicine,* 209**,** 369-375.

SALONEN, J. T., ALFTHAN, G., HUTTUNEN, J. K., PIKKARAINEN, J. & PUSKA, P. (1982) Association between cardiovascular death and myocardial infarction and serum selenium in a matched-pair longitudinal study. *Lancet,* 2**,** 175-179.

SCF, E. (2000) *Opinion of the Scientific Committee on Food on the tolerable upper intake level of selenium*, European Commission.

SCHARPF, M., SCHWEIZER, U., ARZBERGER, T., ROGGENDORF, W., SCHOMBURG, L. & KOHRLE, J. (2007) Neuronal and ependymal expression of selenoprotein P in the human brain. *Journal of Neural Transmission,* 114**,** 877-884.

SCHRAUZER, G. (2000) Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *Journal of Nutrition,* 130**,** 1653-1656.

SCHRAUZER, G. (2003) The nutritional significance, metabolism and toxicology of selenomethionine. *Advances in Food and Nutrition Research.*

SCHWARTZ, T. R. & KMIEC, E. B. (2007) Reduction of gene repair by selenomethionine with the use of single-stranded oligonucleotides. *BMC Mol Biol,* 8**,** 7.

SCHWARZ, K., BIERI, J. G., BRIGGS, G. M. & SCOTT, M. L. (1957) Prevention of exudative diathesis in chicks by factor 3 and selenium. *Proceedings of the Society for Experimental Biology and Medicine,* 95**,** 621-625.

SCHWARZ, K. & FOLTZ, C. (1957) Selenium as an intergral part of factor 3 against dietary necrotic liver degeneration. *Journal of the American Chemical Society,* 79**,** 3292.

SHEN, C., SONG, W. & PENCE, B. (2001) Interactions of selenium compounds with other antioxidants in DNA damage and apoptosis in human normal keratinocytes. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 385-390.

SINHA, R. & EL-BAYOUMY, K. (2004) Apoptosis is a critical cellular event in cancer chemoprevention and chemotherapy by selenium compounds. *Current Cancer Drug Targets,* 4**,** 13-28.

SMITH, L., NAGAR, S., KIM, G. & MORGAN, W. (2003) Radiation-induced genomic instability: radiation quality and dose response. *Health Physics,* 85**,** 23-29.

SMITH, M., LANCIA, J., MERCER, T. & IP, C. (2004) Selenium compounds regulate p53 by common and distinctive mechanisms. *Anticancer Research,* 24**,** 1401-1408.

SOKOLOFF, L. (1987) Kashin-Beck disease. *Rheumatic Diseases Clinics of North America,* 13**,** 101-104.

SPALLHOLZ, J. E. (1994) On the nature of selenium toxicity and carcinostatic activity. *Free Radical Biology & Medicine,* 17, 45-64.

SPEIT, G. & HARTMANN, A. (2005) The comet assay: a sensitive genotoxicity test for the detection of DNA damage. *Methods in Molecular Biology,* 291**,** 85-95.

STADTMAN, T. C. (1980) Selenium-dependent enzymes. *Annual Review of Biochemistry,* 49**,** 93-110.

STURUP, S., HAYES, R. B. & PETERS, U. (2005) Development and application of a simple routine method for the determination of selenium in serum by octopole reaction system ICPMS. *Analytical and Bioanalytical Chemistry,* 381**,** 686-694.

SUDRE, P. & MATHIEU, F. (2001) Kashin-Beck disease: from etiology to prevention or from prevention to etiology? *International Orthopaedics,* 25**,** 175-179.

SUETENS, C., MORENO-REYES, R., CHASSEUR, C., MATHIEU, F., BEGAUX, F., HAUBRUGE, E., DURAND, M. C., NEVE, J. & VANDERPAS, J. (2001) Epidemiological support for a multifactorial aetiology of Kashin-Beck disease in Tibet. *International Orthopaedics,* 25**,** 180-187.

SUNDE, R. (1997) *Selenium, in Handbook of nutritionally essential mineral elements,* New York, Marcel Dekker.

SUZUKI, K. T., DOI, C. & SUZUKI, N. (2006) Metabolism of ^{76}Se-methylselenocysteine compared with that of ^{77}Se-selenomethionine and ^{82}Se-selenite. *Toxicology and Applied Pharmacology,* 217**,** 185-195.

TAPIERO, H., TOWNSEND, D. & TEW, K. (2003) The antioxidant role of selenium and seleno-compounds. *Biomedicine and Pharmacotherapy,* 57**,** 134-144.

TARZE, A., DAUPLAIS, M., GRIGORAS, I., LAZARD, M., HA-DUONG, N. T., BARBIER, F., BLANQUET, S. & PLATEAU, P. (2007) Extracellular production of hydrogen selenide accounts for thiol-assisted toxicity of selenite against Saccharomyces cerevisiae. *Journal of Biological Chemistry,* 282**,** 8759-8767.

TAYLOR, E. W., COX, A. G., ZHAO, L., RUZICKA, J. A., BHAT, A. A., ZHANG, W., NADIMPALLI, R. G. & DEAN, R. G. (2000) Nutrition, HIV, and drug abuse: the molecular basis of a unique role for selenium. *Journal of Acquired Immune Deficiency Syndromes,* 25 Suppl 1**,** S53-61.

THIRUNAVUKKARASU, C. & SAKTHISEKARAN, D. (2003) Sodium selenite, dietary micronutrient, prevents the lymphocyte DNA damage induced by N-nitrosodiethylamine and phenobarbital promoted experimental hepatocarcinogenesis. *Journal of Cellular Biochemistry,* 88**,** 578-588.

THOMSON, C. (2004a) Assessment of requirements for selenium and adequacy of selenium status: a review. *European Journal of Clinical Nutrition,* 58**,** 391-402.

THOMSON, C., ONG, L. & ROBINSON, M. (1985) Effects of supplementation with high-selenium wheat bread on selenium, glutathione peroxidase and related enzymes in blood components of New Zealand residents. *American Journal of Clinical Nutrition,* 41**,** 1015-1022.

THOMSON, C., ROBINSON, M., BUTLER, J. & WHANGER, P. (1993) Long-term supplementation with selenate and selenomethionine: selenium and glutathione peroxidase (EC 1.11.1.9) in blood components of New Zealand women. *British Journal of Nutrition,* 69**,** 577-588.

THOMSON, C. D. (2004b) Selenium and iodine intakes and status in New Zealand and Australia. *British Journal of Nutrition,* 91**,** 661-672.

TINGGI, U., REILLY, C. & PATERSON, C. (1992) Determination of selenium in foodstuffs using spectrofluorometry and hudride generation atomic absorption spectrometry. *Journal of Food Composition and Analysis,* 5**,** 269-280.

UJIIE, S. & KIKUCHI, H. (2002) The relation between serum selenium value and cancer in Miyagi, Japan: 5-year follow up study. *Tohoku Journal of Experimental Medicine,* 196**,** 99-109.

VAN DEN BRANDT, P. A., ZEEGERS, M. P., BODE, P. & GOLDBOHM, R. A. (2003) Toenail selenium levels and the subsequent risk of prostate cancer: a prospective cohort study. *Cancer Epidemiology, Biomarkers and Prevention,* 12**,** 866-871.

VAN DER TORRE, H., VAN DOKKUM, W., SCHAAFSMA, G., WEDEL, M. & OCKHUIZEN, T. (1991) Effect of various levels of selenium in wheat and meat on blood Se status indices and on Se balance in Dutch men. *British Journal of Nutrition,* 65**,** 69-80.

VILLETTE, S., BERMANO, G., ARTHUR, J. R. & HESKETH, J. E. (1998) Thyroid stimulating hormone and selenium supply interact to regulate selenoenzyme gene expression in thyroid cells (FRTL-5) in culture. *FEBS Letters,* 438**,** 81-84.

WATERS, D., SHEN, S., COOLEY, D., BOSTWICK, D., QIAN, J., COMBS, G., JR., GLICKMAN, L., OTEHAM, C., SCHLITTLER, D. & MORRIS, J. (2003) Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. *Journal of the National Cancer Institute,* 95**,** 237-241.

WEI, W. Q., ABNET, C. C., QIAO, Y. L., DAWSEY, S. M., DONG, Z. W., SUN, X. D., FAN, J. H., GUNTER, E. W., TAYLOR, P. R. & MARK, S. D. (2004) Prospective study of serum selenium concentrations and esophageal and gastric cardia cancer, heart disease, stroke, and total death. *American Journal of Clinical Nutrition,* 79**,** 80-85.

WHANGER, P. (2000) Selenoprotein W: a review. *Cellular and Molecular Life Sciences,* 57, 1846-52.

WHANGER, P., VENDELAND, S., PARK, Y. C. & XIA, Y. (1996) Metabolism of subtoxic levels of selenium in animals and humans. *Annals of Clinical Laboratory Science,* 26**,** 99-113.

WINGLER, K., BOCHER, M., FLOHE, L., KOLLMUS, H. & BRIGELIUS-FLOHE, R. (1999) mRNA stability and selenocysteine insertion sequence efficiency rank gastrointestinal glutathione peroxidase high in the hierarchy of selenoproteins. *European Journal of Biochemistry,* 259**,** 149-157.

WINGLER, K. & BRIGELIUS-FLOHE, R. (1999) Gastrointestinal glutathione peroxidase. *Biofactors,* 10**,** 245-249.

WOLFFRAM, S., BERGER, B., GRENACHER, B. & SCHARRER, E. (1989) Transport of selenoamino acids and their sulfur analogues across the intestinal brush border membrane of pigs. *Journal of Nutrition,* 119**,** 706-712.

XIA, Y., HA, P., HILL, K., BUTLER, J. & WHANGER, P. (2000) Distribution of selenium between fractions in erythrocytes, plasma, hair, and fingernails of Chinese women living in selenium-deficient, -adequate,

and -excessive areas of China. *J. Trace Elem. Exp. Med.,* 13**,** 333-342.

XIA, Y., HILL, K. E., BYRNE, D. W., XU, J. & BURK, R. F. (2005) Effectiveness of selenium supplements in a low-selenium area of China. *American Journal of Clinical Nutrition,* 81**,** 829-834.

YANG, G. & ZHOU, R. (1994) Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China. *Journal of Trace Elements and Electrolytes in Health and Disease,* 8**,** 159-165.

YANG, G. Q., CHEN, J. S., WEN, Z. M., GE, K. Y., ZHU, L. Z., CHEN, X. C. & CHEN, X. S. (1984) The role of selenium in Keshan disease. *Advances in Nutritional Research,* 6**,** 203-231.

YANG, G. Q., GE, K. Y., CHEN, J. S. & CHEN, X. S. (1988) Selenium-related endemic diseases and the daily selenium requirement of humans. *World Review of Nutrition and Dietetics,* 55**,** 98-152.

YANG, G. Q., WANG, S. Z., ZHOU, R. H. & SUN, S. Z. (1983) Endemic selenium intoxication of humans in China. *American Journal of Clinical Nutrition,* 37**,** 872-881.

YANG, G. Q. & XIA, Y. M. (1995) Studies on human dietary requirements and safe range of dietary intakes of selenium in China and their application in the

prevention of related endemic diseases. *Biomedical and Environmental Sciences,* 8**,** 187-201.

YEO, J., CHA, S., CHO, C., KIM, S., CHO, J., BAEK, W., SUH, M., KWON, T., PARK, J. & SUH, S. (2002) Se-methylselenocysteine induces apoptosis through caspase activation and Bax cleavage mediated by calpain in SKOV-3 ovarian cancer cells. *Cancer Letters,* 182**,** 83-92.

YOSHIZAWA, K., WILLETT, W. C., MORRIS, S. J., STAMPFER, M. J., SPIEGELMAN, D., RIMM, E. B. & GIOVANNUCCI, E. (1998) Study of prediagnostic selenium level in toenails and the risk of advanced prostate cancer. *Journal of the National Cancer Institute,* 90**,** 1219-1224.

YU, M. W., HORNG, I. S., HSU, K. H., CHIANG, Y. C., LIAW, Y. F. & CHEN, C. J. (1999) Plasma selenium levels and risk of hepatocellular carcinoma among men with chronic hepatitis virus infection. *American Journal of Epidemiology,* 150**,** 367-374.

YU, S. Y., ZHU, Y. J. & LI, W. G. (1997) Protective role of selenium against hepatitis B virus and primary liver cancer in Qidong. *Biological Trace Element Research,* 56**,** 117-124.

ZARCINAS, B. A. (1984) Analysis of soil and plant material by inductively coupled plasma - optical emission spectrometry. CSIRO, Division of Soils.

ZEEGERS, M. P., GOLDBOHM, R. A., BODE, P. & VAN DEN BRANDT, P. A. (2002) Prediagnostic toenail selenium and risk of bladder cancer. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 1292-1297.

ZHAI, S. S., KIMBROUGH, R. D., MENG, B., HAN, J. Y., LEVOIS, M., HOU, X. & YIN, X. N. (1990) Kashin-Beck disease: a cross-sectional study in seven villages in the People's Republic of China. *Journal of Toxicology and Environmental Health,* 30**,** 239-259.

ZHOU, N., XIAO, H., LI, T., NUR, E., A, K. & LIU, L. (2003) DNA damage-mediated apoptosis induced by selenium compounds. *Journal of Biological Chemistry,* 278**,** 29532-29537.

2. Aims, Hypotheses and Study Design

2.1 Project aims

The proposed project aimed to determine

1. the concentration range of selenomethionine (Se-met) that prevents DNA damage and is non-toxic;
2. food groups that are correlated with Se status in older South Australian men;
3. the effect of intake of selenium (Se) either as Se-met in process fortified wheat or organic Se in wheat biofortified with Se on Se status, glutathione peroxidase (GPx) activity and DNA damage in older South Australian men.

2.2 Hypotheses

1. Se-met is non-toxic at supra-physiological concentration.
2. Plasma Se is directly correlated with intake of foods rich in Se-met.
3. Selenium from biofortified high-Se wheat is as bioavailable as wheat fortified post-harvest with Se-met.
4. Selenium from biofortified high-Se wheat has the same bioefficacy as wheat fortified post-harvest with Se-met with respect to DNA damage prevention.

2.3 Project design

The research project included three parts: 1) *in vitro* dose-response study for Se-met in cultured human lymphocytes; 2) a dietary Se intake and plasma Se survey study in older men; and 3) an *in vivo* randomised double-blind placebo-controlled intervention trial conducted over 6 months in healthy older South Australian men. Ethics approval was obtained from both the CSIRO (Division of Human Nutrition) and the University of Adelaide Ethics Committees.

2.3.1 In vitro dose-response studies for Se-met in cultured human lymphocytes

The *in vitro* dose-response studies for Se-met in cultured human lymphocytes aimed to:

1. define the safe dose-range from the point of view of genotoxicity and cytotoxicity;
2. determine the optimal concentration of Se-met for reduction of chromosomal and/or DNA damage;

3. determine the optimal concentration of Se-met for improving resistance to hydrogen peroxide or gamma radiation-induced genome damage.

Primary lymphocytes were isolated from 6 healthy males aged between 28 to 51 years. Cells were cultured in medium supplemented with Se-met in a series of Se concentrations (3, 31, 125, 430, 1880 and 3850 µg Se/l) for 9 days. Chromosomal/ DNA damage was measured at baseline, and following hydrogen peroxide or gamma radiation challenge.

2.3.2 Dietary Se intake and plasma Se survey studies

The dietary Se intake and plasma Se survey studies aimed to:

1. obtain knowledge on the Se intake of the study population by conducting a food frequency questionnaire (FFQ) survey in older South Australian men;
2. obtain knowledge on the Se status of the study population by conducting a plasma Se survey in older South Australian men;
3. identify the food groups and nutrients that are associated with Se intake or Se status;
4. evaluate the correlation between biomarkers of Se status and the intake of Se from foods estimated using CSIRO FFQ.

At the recruitment stage of the intervention study, baseline plasma Se level of the volunteers was determined, two versions of food frequency questionnaire (FFQ), namely CSIRO Dietary Intake Questionnaire (2004) and Anti-Cancer Council Victoria Dietary Questionnaire (2003), were completed by 179 volunteers to estimate their dietary intake of Se.

2.3.3 In vivo randomised double-blinded placebo-controlled intervention trial

The *in vivo* randomised double-blinded placebo-controlled intervention trial aimed to:

1. compare the bioavailability and bioefficacy of Se from biofortified high-Se (BIOFORT) wheat and from wheat process-fortified post-harvest with L-selenomethionine (PROFORT);
2. determine the optimal organic Se intake for prevention of genome damage in a cohort of healthy older men.

This study was designed as a randomised, double-blind, placebo-controlled clinical trial with a dose-response over a 24-week period (Figure 2-1). 179 healthy males were

recruited and screened for plasma Se and those with lowest plasma Se (<100 µg /l) (n=81) were randomised to three treatment groups, namely CONTROL, BIOFOR and PROFORT.

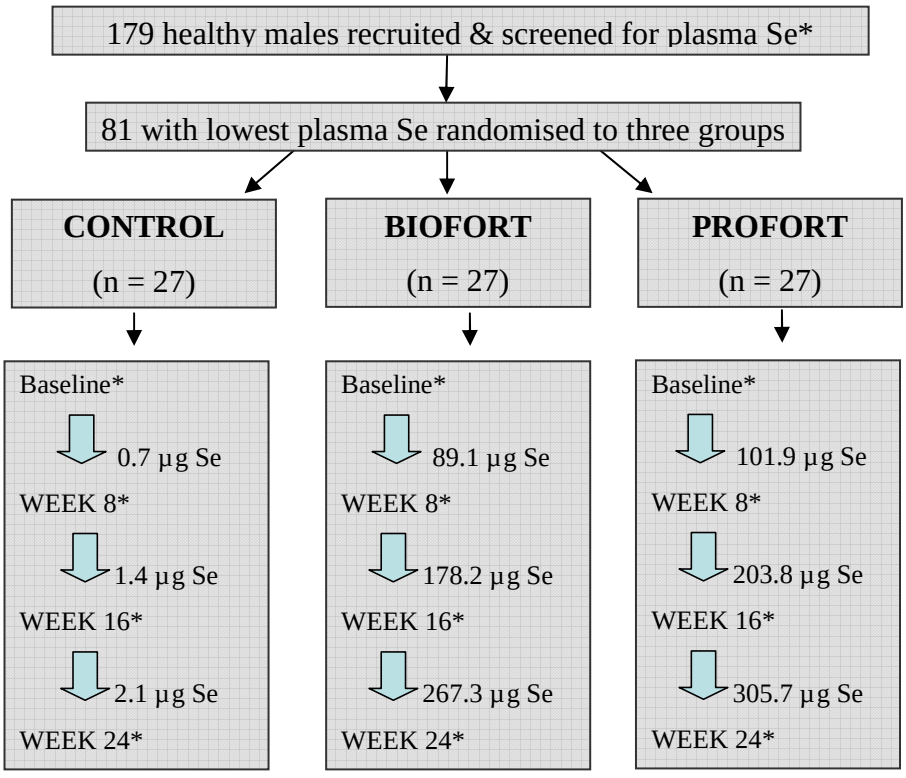
Inclusion criteria:

- Healthy males aged 40-70yr
- Smoker or non-smoker
- Not supplementing with selenium
- Not supplementing with above-RDI levels of folate, vitamin B12, vitamin C

Exclusion criteria:

- Cancer patients undergoing chemotherapy or radiotherapy
- Sensitivity to study foods, i.e. gluten/wheat intolerance
- Unable to comprehend or comply with study protocol
- Not available for all sampling phases of the study

The association of DNA damage with 1) the dietary intake of nutrients; 2) the intake of food groups and 3) plasma concentration of minerals in this group of healthy older South Australian men was also investigated.



* Blood samples collected at the beginning of the indicated week.

Figure 2-1 Trial design

2.4 Outcome measures

2.4.1 Plasma Se

Plasma Se concentration was measured as indicator of Se status as well as a biomarker of Se bioavailability in supplementation. Plasma Se analysed by the South Australia Research and Development Institute (SARDI) was determined by using the fluorometric method. The basis of this method is that the reaction of 2, 3-diaminonaphthalene (DAN) with Se (IV) forms a fluorescent Se-DAN complex, piazselenol (Koh and Benson, 1983). DAN has great fluorescence sensitivity and is extractable into organic solvents from acid solution (Watkinson, 1966). After a plasma sample is digested with nitric acid and perchloric acid, Se is reduced to Se (IV), DAN is added to the sample and followed by extraction using the organic solvent cyclohexane under laboratory fluorescent light. The fluorescence is then measured in the fluorometer (excitation wave length 364 nm and emission wave length 523 nm) and Se concentration is calculated against a series of standards (Koh and Benson, 1983). Selenium concentration in plasma or culture medium measured by Waite Analytical Services (WAS, Adelaide University) was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent Technologies 7500c, Japan), following digestion with nitric acid and hydrochloric acid (Sturup et al., 2005).

2.4.2 GPx activity in platelets

GPx activity in platelets is a functional biomarker of Se status. The change in GPx activity in platelets following Se supplementation can reflect the bioefficacy of Se from supplementation, when plasma Se concentration is lower than 100 µg/l. GPx activity is usually measured by using a commercial GPx activity kit involving an indirect, coupled procedure. GPxs catalyse the reduction of hydroperoxides by reduced glutathione (GSH). Oxidized glutathione (GSSG) is recycled to its reduced state by glutathione reductase and NADPH (reduced nicotinamide adenine dinucleotide phosphate) (Figure 2-2). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in absorbance at 340 nm is directly proportional to the GPx activity in the sample. The GPx activity is expressed in Unit/mg protein. Unit, IU, is equivalent to 1nmol of NADPH oxidised per minute (Reilly, 1996). The quantity of protein in platelet samples was determined by using the Lowry method (Lowry et al., 1951). This is a protein-dye binding method in which a differential change in dye colour occurs in

response to various concentrations of protein. Shift in absorbance is recorded by a microplate reader.

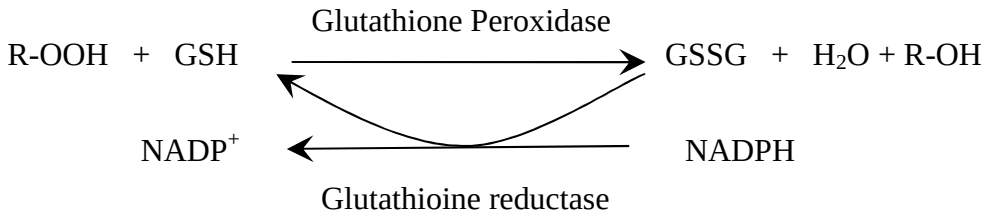


Figure 2-2 GPx activity kit, indirect coupled reaction. Reduction of hydroperoxide is catalysed by GPx at the expense of reduced GSH; oxidized GSSG is recycled to its reduced form at the expense of NADPH catalysed by glutathione reductase (Reilly, 1996)

2.4.3 The CBMN Cyt assay

In the CBMN Cyt assay, a range of chromosomal/DNA damage and cell death biomarkers are measured (Fenech, 2007). Briefly, cultured lymphocytes that have completed one round of nuclear division are blocked from cell division by the addition of Cytochalasin B (Cyto-B), which inhibits cytokinesis without interfering with nuclear division. Cells are harvested onto microscopy slides using a cytocentrifuge. Slides are then air-dried, fixed and stained for visual scoring. DNA damage is expressed in different nuclear dysfunction endpoints in binucleated cells. Micronuclei (MN) are a biomarker of chromosome breakage and whole chromosome loss (Fenech, 1993). Nucleoplasmic bridges (NPBs) are a biomarker of DNA double strand break misrepair, chromosomal rearrangement and potential chromosome telomere end fusion (Fenech and Crott, 2002; Thomas et al., 2003). Nuclear bud (NBud) may arise from the elimination of amplified DNA and possibly elimination of DNA-repair complex (Fenech and Crott, 2002; Lindberg et al., 2007). The frequency of MN, NPB, NBud and micronucleated (MNed) cells per 1000 binucleated cells are calculated as genome toxicity indices. The ratios between mononucleate, binucleate, multinucleate, viable cells are used to calculate the nuclear division index (NDI) as a measure of cell proliferation, mitogen response and cytostatic effects (Fenech, 2007). The proportion of apoptotic and necrotic cell provides a measure of cell death.

2.4.4 The alkaline Comet assay

The alkaline Comet assay enables measurement and visualization of various DNA damage events, as DNA migration, in individual mammalian cells, including the direct breakage of DNA strands (single or double), transient repair sites, alkali-labile sites or abasic sites (Moller, 2005). Briefly, cells are suspended in a low-melting agarose gel

solution on a slide. The slides are put in lysis solution allowing the removal of all cellular proteins. DNA is then subsequently allowed to unwind in alkaline electrophoresis buffer. Abasic alkaline labile sites, including those generated by DNA repair endonuclease, are expressed as single strand breaks (SSB). During the alkaline electrophoresis, DNA being negatively charged is attracted to the anode, but only segments of unwound DNA are free to migrate, extending from the nucleoid head to form the tail of a comet-like image. After neutralisation for a brief period, slides are stained with a fluorescent dye, such as propidium iodide. The comet images thus formed are scored visually under a fluorescent microscope. Quantification is achieved by scoring of 100 randomly selected comets per sample area, assigning them to one of five DNA damage categories, and converting the results to arbitrary units (Collins, 2002; Tice et al., 2000).

2.4.5 Food Frequency Questionnaire (FFQ) survey

Dietary intake of nutrients is usually estimated by the survey method, i.e. using a food frequency questionnaire (FFQ), and the data converted into nutrient intake by using a food composition table. This is an economical procedure compared with direct measurements involving analyses of food actually consumed (Margrettes and Nelson, 1997).

2.5 References

ANTI-CANCER COUNCIL VICTORIA (2003) ACCV Dietary Questionnaire. Victoria, Anti-Cancer Council Victoria.

COLLINS, A. (2002) The Comet Assay - principles, applications, and limitations. IN DIDENKO, V. V. (Ed.) *Methods in molecular biology, in situ detection of DNA damage, methods and protocols.* Totowa, New Jersey, Humana Press.

CSIRO (2004) Selenium Study 2004 - Dietary Intake Questionnaire. Adelaide, CSIRO Health Sciences and Nutrition.

FENECH, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nature Protocol*, 2**,** 1084-1104.

FENECH, M. & CROTT, J. W. (2002) Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research,* 504**,** 131-136.

KOH, T.-S. & BENSON, T. (1983) Critical re-appraisal of fluorometric method for determination of selenium in biological materials. *Analytical Chemistry*, 66**,** 918-926.

LINDBERG, H. K., WANG, X., JARVENTAUS, H., FALCK, G. C., NORPPA, H. & FENECH, M. (2007) Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. *Mutation Research,* 617**,** 33-45.

LOWRY, O., ROSEBROUGH, N., FARR, A. & RANDALL, R. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry,* 193**,** 265-275.

MARGRETTES, B. & NELSON, M. (1997) *Design concepts in nutritional epidemiology,* Oxford, New York, Oxford Medical Publications.

MOLLER, P. (2005) Genotoxicity of environmental agents assessed by the alkaline comet assay. *Basic Clin Pharmacol Toxicol,* 96 Suppl 1**,** 1-42.

REILLY, C. (1996) *Selenium in food and health,* London & New York, Blackie Academic & Professional.

STURUP, S., HAYES, R. B. & PETERS, U. (2005) Development and application of a simple routine method for the determination of selenium in serum by octopole reaction system ICPMS. *Analytical and Bioanalytical Chemistry,* 381**,** 686-694.

THOMAS, P., UMEGAKI, K. & FENECH, M. (2003) Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. *Mutagenesis,* 18**,** 187-194.

TICE, R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. & SASAKI, Y. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* 35**,** 206-221.

WATKINSON, J. (1966) Fluorometric determination of selenium in biological material with 2,3-diaminonaphthalene. *Analytical Chemistry,* 38**,** 92-97.

3. The Effect of Selenium (as Selenomethionine) Concentration on Genome Stability and Cytotoxicity in Human Lymphocytes Measured Using the Cytokinesis-block Micronucleus Cytome Assay

3.1 Summary

A supra-nutritional intake of selenium (Se) may be required for cancer prevention, but an excessively high dose could be toxic. Therefore, the effect on genome stability of L-selenomethionine (Se-met), the most important dietary form of Se, was measured to determine its bio-efficacy and safety limit. Peripheral blood lymphocytes were isolated from six volunteers and cultured with medium supplemented with Se-met in a series of Se concentrations (3, 31, 125, 430, 1880 and 3850 µg Se/l) while keeping the total methionine (i.e. Se-met + L-methionine) concentration constant at 50 µM. Baseline genome stability of lymphocytes and the extent of DNA damage induced by 1.5 Gy γ-ray was investigated using the Cytokinesis-block Micronucleus Cytome (CBMN-Cyt) assay after 9 days of culture in 96-microwell plates. High Se concentrations (≥1880 µg Se/l) caused strong inhibition of cell division and increased cell death ($P<0.0001$). Baseline frequency of nucleoplasmic bridges (NPBs) and nuclear buds (NBud) however, declined significantly (P trend < 0.05) as Se concentration increased from 3 µg Se/l to 430 µg Se/l. Se concentration (≤ 430 µg Se/l) had no significant effect on baseline frequency of micronuclei (MN) and had no protective effect against genome damage induced by exposure to 1.5 Gy γ-ray irradiation. In conclusion, Se, as Se-met, may improve genome stability at concentrations up to 430 µg Se/l, but higher doses may be cytotoxic. Therefore, a cautious approach to supplementation with Se-met is required to ensure that optimal genome health is achieved without cytotoxic effects.

3.2 Introduction

In the past few decades, selenium (Se), which is an essential micronutrient, has attracted much attention due to its cancer-chemopreventive properties in human health (Combs, 2005; Papp et al., 2007). Se exerts its biological functions through more than 20 selenoproteins in humans, such as glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) (Arthur, 2000; Hatfield et al., 2006). Recent studies suggested that Se may reduce the risk of some cancers, particularly prostate cancer (Clark et al., 1998; Duffield-Lillico et al., 2003; Etminan et al., 2005). However, a supra-nutritional intake of Se or a plasma Se level of at least 120 µg Se/l may be required for reducing cancer risk (Combs, 2001a).

Increased genome instability is considered one of the fundamental causes of cancers (Ames et al., 1993; Fenech, 2008). Se plays an important role in protection of DNA from oxidative damage through selenoprotein glutathione peroxidases (GPxs), which are scavengers of reactive oxygen species (ROS) (Arthur, 2000). However, the effect of Se on chromosomal stability remains largely unknown. L-selenomethionine (Se-met) is one of the most important dietary forms of Se in humans and therefore is commonly used in cancer prevention studies (Ip et al., 2000). However, excessive intake of Se-met is toxic (Schrauzer, 2000) but its upper safety limit is not well defined currently.

The cytokinesis-block micronucleus cytome (CBMN-Cyt) assay is one of the best validated cytogenetic tests that can be used to measure chromosome breakage, DNA mis-repair that leads to major chromosome rearrangements such as misrejoining of double strand breaks (DSBs), and chromosome malsegregation as well as necrosis, apoptosis and cytostasis (Fenech, 2007). With good reliability and reproducibility, this assay is widely used in the pharmaceutical industry as the gold standard of genotoxicity testing of clastogenic agents, in human biomonitoring of environmental exposures, preventive medicine and nutritional efficacy (Fenech, 2007). The genome damage biomarkers measured in the CBMN-Cyt assay in lymphocytes were shown to be either predictive of cancer risk in human populations or identified those smokers with a high risk of developing lung cancer in a case-control study (El-Zein et al., 2006; Bonassi et al., 2007). Therefore, this assay is a valid tool in studying the cytogenetic and potential cancer-protective effect of Se-met in human peripheral blood lymphocytes.

The present study tested the genome toxicity and cytotoxicity effect of Se-met at a range of physiologically relevant concentrations of Se using a primary lymphocyte long-term culture model that previously had been shown to be sensitive to the genome damaging effects of folate deficiency within the physiological range (Kimura et al., 2004; Wang et al., 2004). In this study we aimed to 1) define the safe dose-range from the point of view of genotoxicity and cytotoxicity; 2) determine the optimal concentration of Se-met for reduction of chromosomal damage and 3) determine the optimal concentration of Se-met for improving resistance to gamma radiation-induced genome damage.

3.3 Study design and methods

3.3.1 Recruitment

Six male volunteers were recruited from staff members of CSIRO Human Nutrition or University of Adelaide after giving informed consent. Inclusion criteria were: (1) healthy males aged 18-65 yrs; (2) not supplementing with Se or having refrained from supplementation for 4 weeks if taking >2x recommended dietary intake of Se supplement and (3) not taking above the recommended daily intake (RDI) levels of folate, vitamin B_{12}, vitamin C per day on average or having refrained from supplementation for 1 week prior to giving blood. Ethics approval was obtained from the Human Research Ethics Committee of CSIRO Human Nutrition.

Volunteers were required to complete a standard general lifestyle questionnaire while attending the clinic, in which details of any supplements taken were recorded, such as product brand, active ingredients per tablet, dose and frequency of intake. In addition, the quantity and frequency of Brazil nut consumption was also determined because these nuts are usually rich in Se (Reeves et al., 2007).

3.3.2 Collection of blood samples and determination of Se

For each volunteer, 18 ml of whole blood was collected by venipuncture into lithium-heparin vacutainer tubes (Vacuette, Austria). Blood was taken between 8 am – 11 am after an overnight fast and with only a light breakfast. (i.e. a slice of toast without butter and one cup of coffee/tea with low-fat milk). Two ml of plasma was obtained from each sample by centrifuging 5 ml of whole blood at 3000 rpm for 20 min. Plasma Se level was measured at Waite Analytical Services (WAS) using inductively coupled plasma

mass spectrometry (ICP-MS) after acid digestion (Zarcinas et al., 1987; Zarcinas et al., 1996).

3.3.3 Lymphocyte isolation

12 ml of the blood sample was diluted with an equal volume of Hanks balanced salt solution (HBSS, Thermo, US) and mixed. To isolate lymphocytes, 8 ml Ficol Paque (Amersham Pharmacia Biotech, Sweden) was added to a 50 ml Falcon tube and 24 ml of diluted blood was gently overlaid. The tubes were centrifuged at 400 g for 30 min at 22^{o}C. The cells were collected from the fluffy layer and washed with 3 volumes of HBSS, then centrifuged at 180 g for 10 min at room temperature. The cell pellet in the Falcon tube (BD, Australia) was resuspended in 18 ml of HBSS and 3 ml of the cell suspension was transferred into each of 6x TV-10 tubes (Techno Plas, Australia). The cells were washed once more in HBSS and the cell pellet was resuspended in 1 ml of appropriate Se-supplemented culture medium containing 10% foetal bovine serum (FBS, Trace MultiCel, Australia). The concentration of cells was adjusted to 1 x 10^6 cells/ml, then further diluted to obtain 6 ml of 1.5 x 10^5 cells/ml suspension with appropriate culture medium according to the cell count results.

3.3.4 Cell counting using the Coulter Counter

Cell count was measured using a Coulter Counter (Model Z1, Beckman, Australia). The count of each sample was taken in triplicate and the mean value of samples containing cells measured. The mean of blank counts was subtracted from the mean cell count before calculating the cell concentration. Cell concentration (cells/ml) was determined by multiplying the value by 2000 (dilution factor 1000 and counting volume of 0.5 ml).

3.3.5 Culture medium

Se-met supplemented culture medium was prepared using Se-deficient RPMI-1640 (Sigma, Australia) medium deficient in L-methionine, L-cystine, L-glutamine and selenium, to which 10% FBS (Trace MultiCel, Australia) was added. Se concentration in FBS was 13 µg Se/l and therefore effectively only contributed 1.3 µg Se/l to the medium. L-glutamine (Sigma, Australia) and L-cystine (Sigma, Australia) were added to achieve final concentrations of 2.0 mM and 0.27 mM, respectively, to match with the components and concentrations in a standard RPMI-1640 recipe. The final 50 µM total methionine concentration (either L-methionine or L-selenomethionine or a combination of both, Sigma, Australia) was chosen because it sustains cell division in vitro in long term cultures and is close to the physiological range in plasma *in vivo* (20-30 µM)

(Kimura et al., 2004). Using different L-selenomethionine/methionine (without Se) ratios, it was possible to construct six different culture media that only differed in the content of Se and were otherwise identical in total methionine (Se-met plus L-methionine content) concentration. The intended and actual concentrations measured by ICP-MS (shown in brackets) were 1 (3), 120 (125), 480 (430), 1920 (1880) and 3950 (3850) µg Se/l. The highest concentration represented the situation for all of methionine being supplied as Se-met; i.e. 3950 µg Se/l is equivalent to 50 µM Se-met. For the purpose of this study, Se-met concentration is shown as µg Se/l to enable comparison with the conventional manner of expressing Se concentration *in vivo*. For conversion to µM values, the following equation is used: 100 µg Se (as Se-met) = 1.25 µM Se-met.

Calculations for preparing a total final volume of 117.6 ml culture medium with 6 different Se concentrations are specified in Table 3-1 and Table 3-2.

Table 3-1 Se concentrations in culture medium

Treatments	A	B	C	D	E	F
Intended final Se in medium (µg/l)	1	30	120	480	1920	3950
Actual measured Se in medium (µg/l)	3	31	125	430	1880	3850
Intended final Se-met in medium (µM)	0	0.4	1.5	6.0	24.1	50
Intended final met in medium (µM)	50	49.6	48.5	44.0	25.9	0

Se-met, L-selenomethionine; met, L-methionine.

Table 3-2 Preparation of culture medium with varying Se concentrations

Treatments	A	B	C	D	E	F
RPMI-1640 deficient medium * (ml)	95.3	95.3	95.3	95.3	95.3	95.3
FBS (10% final) (ml)	11.8	11.8	11.8	11.8	11.8	11.8
100 mM Sodium pyruvate solution ** (ml)	1.18	1.18	1.18	1.18	1.18	1.18
Pen/Strep antibiotic solution *** (ml)	1.18	1.18	1.18	1.18	1.18	1.18
29.2 mg/l stock L-glutamine solution (ml)	1.18	1.18	1.18	1.18	1.18	1.18
5.9 mg/l stock L-cystine.2HCl solution (ml)	1.18	1.18	1.18	1.18	1.18	1.18
298.4 µg/l stock L-methionine solution (µl)	2941	2920	2853	2585	1512	0
196.1 µg/l stock L-selenomethionine solution (µl)	0	43	177	713	2859	5883
Extra RPMI-1640 to make up 117.6 ml (µl)	2941	2920	2853	2585	1512	0

* RPMI-1640 medium was deficient in L-glutamine, L-cystine and L-methionine – the stock solutions of these agents were prepared in the RPMI-1640 medium and sterilized using 0.22 µm filter.

** Sodium pyruvate (Sigma) was purchased as a sterile 100 mM solution in saline, final concentration in culture medium = 1 mM.

*** Penicillin/Streptomycin (Sigma) was purchased as a sterile solution of 5000 IU/ml Penicillin / 5000 µg/ml Streptomycin in tissue culture grade water, final concentration in culture medium 50 IU Penicillin / 50 µg/ml Streptomycin.

3.3.6 9-day lymphocyte culture in 96-microwell plate

Isolated lymphocytes were cultured in 96-microwell plates (NUNC flat bottom, sterile with lid, 200 µl per well) with appropriate seleno-L-methionine supplemented culture medium at a concentration of 1.5 x 10^5 cells/ml in 200 µl. Phytohaemagglutinin (PHA, Abbott Murex #HA15 8E27, Australia) and Interleukin-2 (IL-2, Roche Diagnostics Cat# 799068, Australia) were added to stimulate cell division and growth using a final concentration of PHA of 30 µg/ml and 4 units of IL-2 per 200 µl culture volume in each well. The seeding concentration of lymphoyctes was determined in preliminary experiments aimed at optimising percentage of binucleated cells accumulated 24 hours following Cytochalasin-B (Cyto-B, Sigma, Australia) treatment on day 9 (Figure 3-1). Time of adding PHA was recorded on day 0. Cells were cultured in a humidified incubator at 37^{o}C with 5% CO_2 (Sanyo MCO-17 A1C, Japan) for 6 days. On day 7, half of the culture medium was removed carefully without disturbing the cells and replaced with fresh culture medium with IL-2 and Se-Met at the same concentration used on day 0. Cells were cultured for a further 2 days. These experiments were performed (once in duplicate) for lymphocytes from six different individuals. Cultured cells from wells of the same Se concentration were pooled together into a TV-10 tube on day 9 and the cell count was measured. CBMN-Cyt assay was performed on day 9 to assess genome damage of cultured lymphocytes. Based on an average cell cycle time of 17 hours for lymphocytes grown in RPMI 1640 medium (Henderson et al., 1997) we estimate that the dividing fraction of the cultures would have undergone approximately 13 cell divisions over the 10 day culture period.

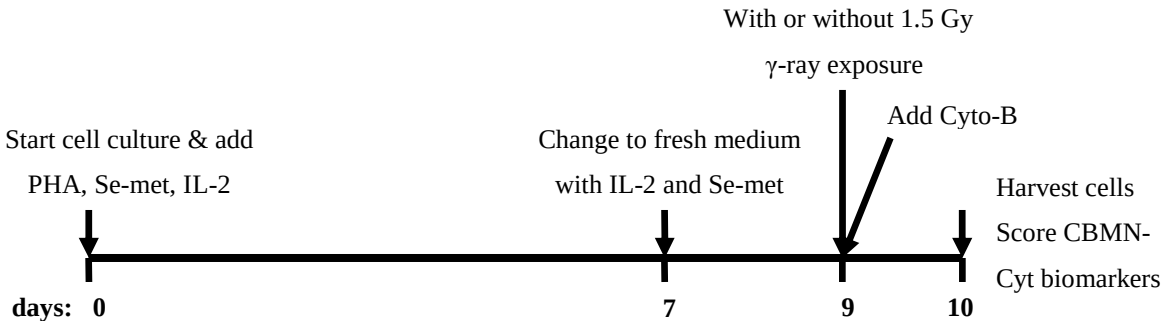


Figure 3-1 Schematic diagram of protocol of long-term culture and CBMN-Cyt assay for testing cytotoxic and genotoxic effects of Se-met concentration.

3.3.7 γ-Ray- irradiation of cells

Two aliquots of cell suspension were pooled from each of the six Se cultures and transferred into TV-10 tubes on day 9 in the morning. They were challenged with 1.5 Gy γ-ray radiation using a CIS BIO International IBL 437 blood cell irradiator

comprising three stationary ^{137}Cs doubly encapsulated radiation sources emitting γ-radiation at 5.3 Gy/min located at the Transfusion Medicine Unit, IMVS (Institute of Medical and Veterinary Science). An exposure of 17 sec is equivalent to 1.5 Gy γ irradiation. Post-irradiation, cells were incubated at 37^{o}C for 30 min before adding Cytochalasin-B (Cyto-B). Control (untreated) cells were treated in the same way but were not exposed to γ-rays.

3.3.8 CBMN-Cyt assay

3.3.8.1 CBMN Cyt assay

Lymphocytes that were either unexposed or exposed to 1.5Gy γ–radiation on day 9 were resuspended in culture medium and cultured in the presence of Cyto-B (4.5 µg/ml) in TV-10 tubes (37^{o}C, 5% CO_2) for 24 hours. Cells were then harvested onto microscope slides using a cytocentrifuge (Shandon Products, UK). The slides were air-dried for 15 min, fixed in absolute methanol for 10 min and stained using Diff-Quik stains (Lab Aids, Australia). Slides were prepared in duplicate.

For each Se concentration/treatment/individual, a total of 2000 binucleated (BN) cells were scored for the presence of micronuclei (MN, biomarker of chromosome breakage or loss), nucleoplasmic bridges (NPB, biomarker of asymmetrical chromosome rearrangement) and nuclear buds (NBud, biomarker of gene amplification) (Fenech, 2007). The frequency of BN cells with MN (BN-MN), BN cells with NPB (BN-NPB) and BN cells with NBud (BN-NBud) was measured to determine genome damage indices.

Three hundred cells were scored and classified to determine ratios of mononucleate, binucleate, multinucleate, apoptotic and necrotic nuclei. These ratios were used to calculate percentage of necrotic and apoptotic cells as well as the nuclear division index (NDI). NDI was measured to determine cytostatic effects and the rate of mitotic division. NDI was calculated as follows:

$$NDI = [M1 + 2(M2) + 3(M3) + 4(M4)]/N$$

Where M1 - M4 = the number of cells with one to four nuclei and N = the total number of cells scored (viable and non-viable).

3.3.8.2 Scoring criteria

All slides were coded prior to scoring and the scorer was made unaware of the treatment given to the cells examined by coding of slides by another person who was not working on this project. Scoring was carried out by a single scorer (JW) using a bright field microscope (Leica 20EB) at 100X magnification under oil immersion. Scorer calibration was conducted against an experienced scorer (CS) in our lab. In total, 22 slides (11 samples) were scored. Cells of each sample were cultured in duplicates and one slide was prepared for each culture. The coefficient of variation between scorers and between two cultures of one sample was calculated as shown in Table 3-3. The extent of variation of scores for JW relative to CS was considered to be sufficiently low for JW to proceed with scoring the experiment slides.

Table 3-3 The coefficient of variation between two scorers and between two cultures of one sample on CBMN Cytome assay scoring endpoints.

CBMN Cyt assay biomarkers	CV%		
	Between scorers	Between cultures (JW)	Between cultures (CS)
% BN	1.9	4.5	3.1
% Apoptosis	4.0	63.5	35.4
% Necrosis	15.5	1.7	8.6
MNedBN/1000 cells	14.7	9.0	31.4
MN_in_BN/1000 cells	19.2	10.2	28.3
BN-NPB/1000 cells	37.8	74.8	101.0
BN-NBud/1000 cells	3.21	14.8	47.1

Criteria for scoring MN, NPB, NBud, apoptotic and necrotic cells (Figure 3-2) Figure described by Fenech (Fenech, 2007) were as follows:

<u>1. Criteria for selecting binucleated (BN) cells which can be scored for the presence of micronuclei, nucleoplasmic bridges and nuclear buds</u>

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter.

- The two main nuclei in a BN may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

2. Criteria for scoring micronuclei (MN)

- The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.
- MN are round or oval in shape.
- MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles.
- MN are not linked or connected to the main nuclei.
- MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MN usually has the same staining intensity as the main nuclei but occasionally staining may be more intense.

3. Criteria for scoring nucleoplasmic bridges (NPB)

- NPB is a continuous nucleoplasmic link between the nuclei in a binucleated cell.
- The width of a nucleoplasmic bridge may vary considerably but usually does not exceed one-quarter of the diameter of the nuclei within the cell.
- NPB should have the same staining characteristics of the main nuclei.
- On rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell.
- A binucleated cell with a nucleoplasmic bridge may or may not contain one or more micronuclei.

4. Criteria for scoring nuclear buds (Nbud)

- NBud is an extruded nuclear material with a clearly narrower nucleoplasmic connection (or‘neck’) to the parental nucleus in a binucleated cell.
- There can be more than one bud observed per cell, but it is scored as one BN cell with NBud.

5. Criteria for scoring apoptotic cells

- Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries.
- Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.

- Staining intensity in the nucleus, nuclear fragments and cytoplasm is usually greater than in viable cells.

6. Criteria for necrotic cells

- Early necrotic cells can be identified by the presence of pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus.
- Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.
- Staining intensity of the nucleus and cytoplasm is usually less than that observed in a viable cell.

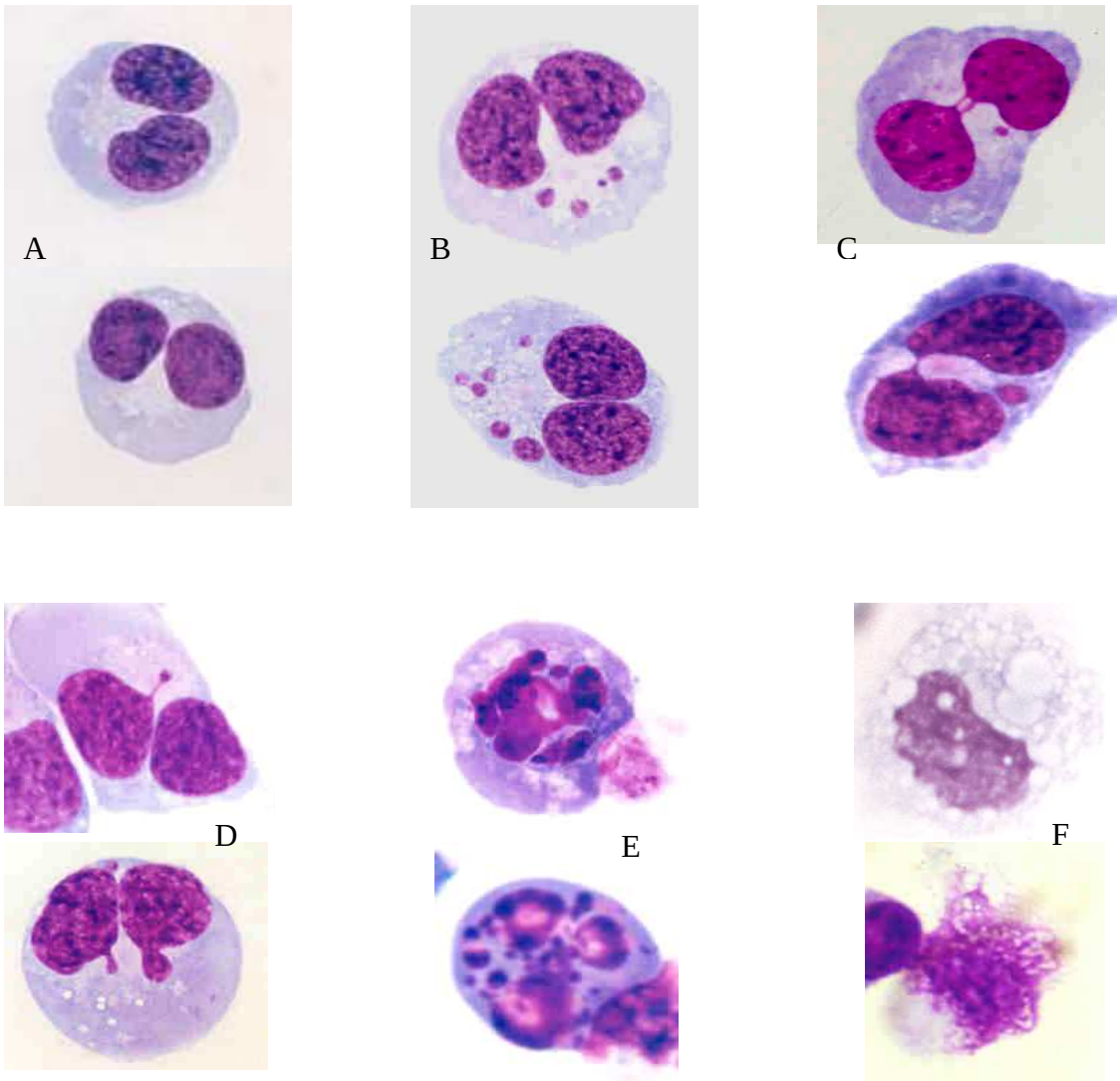


Figure 3-2 Photomicrographs of: A. normal binucleated cells; B. normal binucleated cells with micronuclei; C. normal binucleated cells with nucleoplasmic bridges and micronuclei; D. normal binucleated cells with nuclear buds; E. apoptotic cells; F. necrotic cells.

3.3.9 Statistical analysis

All endpoints measured in this study were tested for Gaussian distribution by using Kolmogorov-Smirnov test. Tests for comparing means of treatment groups were then selected accordingly, i.e. repeated measures one-way ANOVA followed by Tukey's post hoc tests for data with Gaussian distribution and the non-parametric Friedman test followed by Dunn's multiple comparison test for data that did not exhibit Gaussian distribution. Trend tests for relationship of response variables with Se-met doses were also performed. A $P<0.05$ was considered statistically significant. All of the data are expressed as mean ± standard error (SE). Tests were performed using Prism 4.0 (GraphPad Inc., San Diego, CA) software.

3.3.10 Lymphocyte long-term culture pilot study

The aim of this pilot study was to determine the optimal starting cell concentration for a 10-day lymphocyte culture. Frozen human lymphocytes were used in this study.

Cell culture protocol was modified from the standard laboratory protocol (SOP 057/2) for 1 ml isolated lymphocyte culture volume in TV-10 tubes for 200 µl culture volume in 96-microwell plate culture. The final PHA (Abbott Murex #HA15 8E27) concentration was kept the same at 30 µg/ml. However, the concentration of IL-2 (Roche Diagnostics Cat. # 799068) was doubled to 20 unit/ml instead of 10 unit/ml because the lower cell concentration used might lead to inadequate IL-2 production. Further more, IL-2 was added on both day 0 and day 7 when changing the culture medium.

Starting cell concentrations tested were 1×10^5 cells/ml (2×10^4 cells/well), 1.5×10^5 cells/ml (3×10^4 cells/well) and 2×10^5 cells/ml (4×10^4 cells/well). Cells were continuously cultured in 96-microwell plate for 6 days. Half of the culture medium was removed carefully on day 7 without disturbing the cells. Fresh culture medium and IL-2 were added to keep the nutrient and growth factor concentrations up. Cell counts and viability counts were conducted on day 9 and CBMN Cyt assay on days 9 and 10 with Cyto-B addition on day 9 (Table 3-4).

Table 3-4 Cell number, concentration and/or viability on day 0 and day 9

Number of cells per well on day 0	Cell concentration on day 0 (cells/ml)	Cell concentration on day 9 (cells/ml)	Viability on day 9 (%)
2×10^4	1×10^5	0.7×10^6	92.7
3×10^4	1.5×10^5	2.0×10^6	97.6
4×10^4	2×10^5	2.2×10^6	98.3

Microscopical observation showed that cell colonies covered 50-70% of the well bottom in cultures with 2×10^4 cells/well seeding concentration while > 90% of the well bottom was covered by cells in cultures with 3×10^4 cells/well and 4×10^4 cells/well seeding concentrations.

Cyto-B was added to a final concentration of 4.5 µg/ml to the cell cultures on day 9. Cells were harvested 24 hours later. The percentage of binucleated cells in 2×10^4 cells/well culture was about 50-55% while that in 3×10^4 cells/well culture was about 40%. (Note: The slide of 4×10^4 cells/well was not scored because the slide was accidently lost.) Considering the cell number and volume required for both CBMN Cytome assay and Comet assay and practicality of the protocol, the seeding concentration of 2.5×10^4 cells/well was chosen as the optimal choice for the study.

3.4 Results

3.4.1 Plasma Se of volunteers

The characteristics of the volunteers are shown in Table 3-5. The volunteers were healthy non-smoking males aged between 28 and 51 years (mean 42.7 yr). The mean plasma Se was 145.2 ± 20.5 µg Se/l, which indicated that on average the volunteers were replete in Se, assuming 120 µg Se/l as the replete cut-off value based on cancer risk minimisation (Combs, 2001b).

Table 3-5 Characteristics of the volunteers

Volunteer profile	
Volunteers	N = 6
Gender	All male
Age, yr, mean (range)	42.7 (28-51)
Plasma Se (µg Se/l), mean (range)	145.2 (93-240)
Multi-vitamin supplement (Se 25 µg/day)	N = 3
Se supplement (200 µg/day)	N = 1
No Se supplement	N = 2
Brazil nut consumption	N = 0

3.4.2 Effect of Se-met on cell growth and viability on day 9 and day 10

Cell concentrations of cultures on day 9 with various Se doses were significantly different with a peak at 31 µg Se/l and an obvious marked decline at 1880 and 3850 µg Se/l ($P<0.0001$) (Figure 3-3 A). Apoptosis was not markedly affected by Se concentration; however, there was a significant trend for more apoptosis as Se concentration increased up to 3850 µg Se/l (P trend < 0.05) (Figure 3-3 B). Necrosis increased sharply at 1880 and 3850 µg Se/l relative to the lower concentrations ($P<0.0001$) (Figure 3-3 C). In cultures with high Se concentration (1880 and 3850 µg Se/l), there were few cells actively undergoing cell division as evidenced by lack of binucleated cells and very low NDI values (NDI=1.03, 1.0 respectively). There was a significant trend of reduction in NDI ($P<0.0001$) with increased Se concentration (Table 3-6 and Figure 3-3 D).

3.4.3 Effect of Se-met on baseline DNA damage

For cultures exposed to high Se concentrations (1880 and 3850 µg Se/l), it was not possible to determine the frequency of the DNA damage endpoints reliably as there were too few binucleated cells (<3% for 1880 µg Se/l and 0% for 3850 µg Se/l) due to strong cytotoxic and cytostatic effects.

MN-BN appeared to increase at Se concentration of 31 µg Se/l relative to 3 µg Se/l and then to decline to a lower level at 125 µg Se/l and 430 µg Se/l, however, these changes were not significant. The results showed that Se concentration up to 430 µg Se/l had no marked effect on BN-MN (Figure 3-3 E).

There was a significant trend for a reduction in baseline frequency of BN-NPB (P for trend < 0.05) and BN-NBud (P for trend < 0.05) when Se concentration increased from 3 µg Se/l to 430 µg Se/l (Figure 3-3 F-G). The percentage reduction at 430 µg Se/l relative to 3 µg Se/l for BN-NPB was 40% and for BN-NBud, was 70% (Table 3-6).

Table 3-6 Cell growth (on day 9), cytotoxicity biomarkers and frequency of BN cells with MN (BN-MN), BN cells with NPB (BN-NPB) and BN cells with NBud (BN-Nbud) (on day 10) for cells cultured at increasing doses of Se-met.

Endpoint on day 9/10	Concentration of Se (as Se-met) in medium (µg Se/l)*						Friedman or ANOVA P	P-trend
	3	31	125	430	1880	3850		
Cell conc. (10^5 cells/ml)	$4.0 \pm 0.05^{a,b,c}$	7.2 ± 0.11^{a}	$5.4 \pm 0.07^{a,b}$	$5.6 \pm 0.08^{a,b}$	$0.9 \pm 0.01^{b,c}$	0.8 ± 0.01^{c}	**< 0.0001**	**< 0.0001**
% Apop.	0.15 ± 0.1	0.23 ± 0.13	0.12 ± 0.12	0.2 ± 0.1	0.18 ± 0.1	0.9 ± 0.34	0.10	**0.01**
% Necr.	14.5 ± 1.1^{a}	$16.8 \pm 3.2^{a,b}$	10.1 ± 1.5^{a}	10.2 ± 1.3^{a}	24.5 ± 3.8^{b}	35.2 ± 3.0^{c}	**< 0.0001**	**< 0.0001**
NDI	1.22 ± 0.04^{a}	$1.14 \pm 0.01^{a,b}$	1.19 ± 0.02^{a}	1.2 ± 0.03^{a}	$1.03 \pm 0.01^{a,b}$	1.0 ± 0^{b}	**0.0003**	**< 0.0001**
BN-MN/1000 BNCs	10.5 ± 1.5	14.43 ± 1.8	12.8 ± 2.2	10.5 ± 1.3	-	-	0.08	0.77
BN-NPB/1000 BNCs	5.2 ± 1.0	5.0 ± 0.9	3.5 ± 0.9	3.1 ± 1.1	-	-	**0.02**	**0.03**
BN-NBud/1000 BNCs	2.7 ± 0.7^{a}	$1.6 \pm 0.6^{a,b}$	$1.8 \pm 0.2^{a,b}$	0.8 ± 0.3^{b}	-	-	**0.01**	**0.008**

%Apop., percentage of cells that were apoptotic; %Necr., percentage of cells that were necrotic; NDI, nuclear division index; BN-MN: number of binucleated cells with micronuclei; BN-NPB: number of binucleated cells with necleoplasmic bridges; BN-Bud: number of binucleated cells with nuclear buds.

Data were compared across Se concentrations either using either one-way ANOVA with post-hoc Tukey test or Friedman test followed Dunn's multiple comparison test, and test for linear trend. Groups not sharing the same superscript letter are significantly different to each other ($P<0.05$). Results are shown as mean ± SE for cultures from six different subjects.

* 100 µg Se as Se-met = 245 µg Se-met = 1.25 µM Se-met

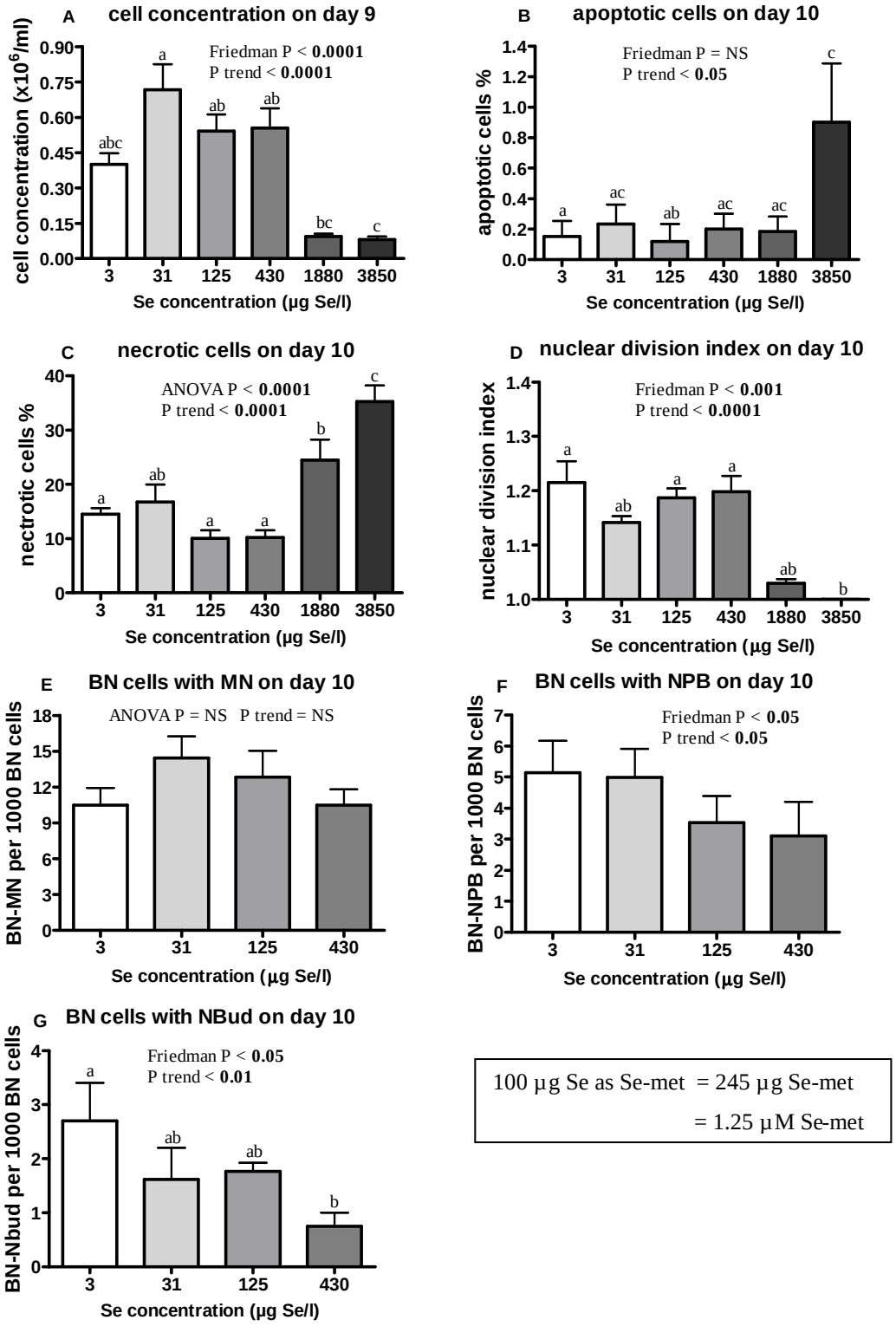


Figure 3-3 Impact of Se concentration on cell growth on day 9 and cytotoxicity and genome damage endpoints scored using CBMN-Cyt assay on day 10: A. cell concentration; B. percentage of apoptotic cells; C. percentage of necrotic cells; D. nuclear division index; E. number of binucleated cells with micronuclei (BN-MN); F. number of binucleated cells with nucleoplasmic bridges (BN-NPB); G. number of binucleated cells with nuclear buds (BN-NBuds). Groups not sharing the same letter are significantly different to each other (P<0.05). Results are shown as mean ± SE for cultures from six different subjects.

3.4.4 Effect of Se-met on γ-ray induced cytotoxicity and DNA damage

To estimate the induced effects of γ-irradiation on cytotoxicity and DNA damage, we subtracted baseline values of control cultures from those of corresponding irradiated cultures.

Se concentration did not affect the percentage of apoptotic cells in γ-irradiated cultures. However, with increasing Se concentration, there was a significant trend of increase in the percentage of γ-irradiation-induced apoptotic cells (P trend < 0.05) (Figure 3-4 A). The percentage of necrotic cells was significantly elevated at 3850 µg Se/l compared to that of lower concentrations, i.e. 31, 125 and 430 µg/l (P<0.05) (Table 3-7). Overall, NDI was reduced by γ irradiation but Se concentration had no impact on NDI in the 3-430 µg Se/l concentration range in γ-irradiated cultures. The apparent lack of effect on NDI by γ irradiation on NDI of 1880 and 3850 µg Se/l was due to the fact that NDI was already minimal (=1) even in non-irradiated cells.

Se at any concentration examined showed no protective effect against γ-ray challenge in terms of induced BN-NPBs and BN-NBuds. The frequencies of induced BN-MN in the 30 µg Se/l cultures were significantly greater than those in 3 µg Se/l (P < 0.05) (Table 3-7 and Figure 3-4 D).

Table 3-7 Cytotoxicity biomarkers and frequency of BN cells with MN (BN-MN), BN cells with NPB (BN-NPB) and BN cells with NBud (BN-Nbud) on day 10 scored using CBMN-Cyt assay in cells exposed to γ irradiation (1.5Gy) in cells cultured at increasing doses of Se-met.

Endpoint on day 10	Concentration of Se (as Se-met) in medium (µg Se/l)*						Friedman or ANOVA P	P-trend
	3	31	125	430	1880	3850		
% Apop.	0.35 ± 0.14	0.15 ± 0.10	0.35 ± 0.14	0.18 ± 0.10	0.15 ± 0.10	0.30 ± 0.15	0.88	0.71
% Necr.	15.77 ± 2.03^{a,b}	13.13 ± 1.95^{a}	12.20 ± 1.89^{a}	13.98 ± 2.26^{a}	23.77 ± 3.32^{a,b}	33.95 ± 2.81^{b}	**0.0005**	**<0.0001**
NDI	1.14 ± 0.01^{a}	1.10 ± 0.01^{a}	1.13 ± 0.01^{a}	1.11 ± 0.02^{a}	1.04 ± 0.01^{b}	1.0 ± 0^{b}	**<0.0001**	**<0.0001**
BN-MN/1000 BNCs	75.95 ± 27.74^{a}	91.87 ± 25.98^{b}	86.0 ± 28.83^{a,b}	82.0 ± 19.5^{a,b}	-	-	**0.005**	0.31
BN-NPB/1000 BNCs	23.47 ± 3.81	22.83 ± 2.43	23.58 ± 3.0	21.82 ± 3.31	-	-	0.996	0.66
BN-NBud/1000 BNCs	3.65 ± 0.85^{a}	1.23 ± 0.59^{b}	2.0 ± 0.74^{a,b}	1.85 ± 0.54^{a,b}	-	-	**0.0015**	**0.001**

% Apop., percentage of cells that were apoptotic; % Necr., percentage of cells that were necrotic; NDI, nuclear division index; BN-MN: number of binucleated cells with micronuclei; BN-NPB: number of binucleated cells with necleoplasmic bridges; BN-Bud: number of binucleated cells with nuclear buds.

Data that were compared across Se concentrations using either one-way ANOVA with post-hoc Tukey test or Friedman test followed Dunn's multiple comparison test, and test for linear trend. Groups not sharing the same superscript letter are significantly different to each other (P<0.05). Results are shown as mean ± SE for cultures from six different subjects.

* 100 µg Se as Se-met = 245 µg Se-met = 1.25 µM Se-met

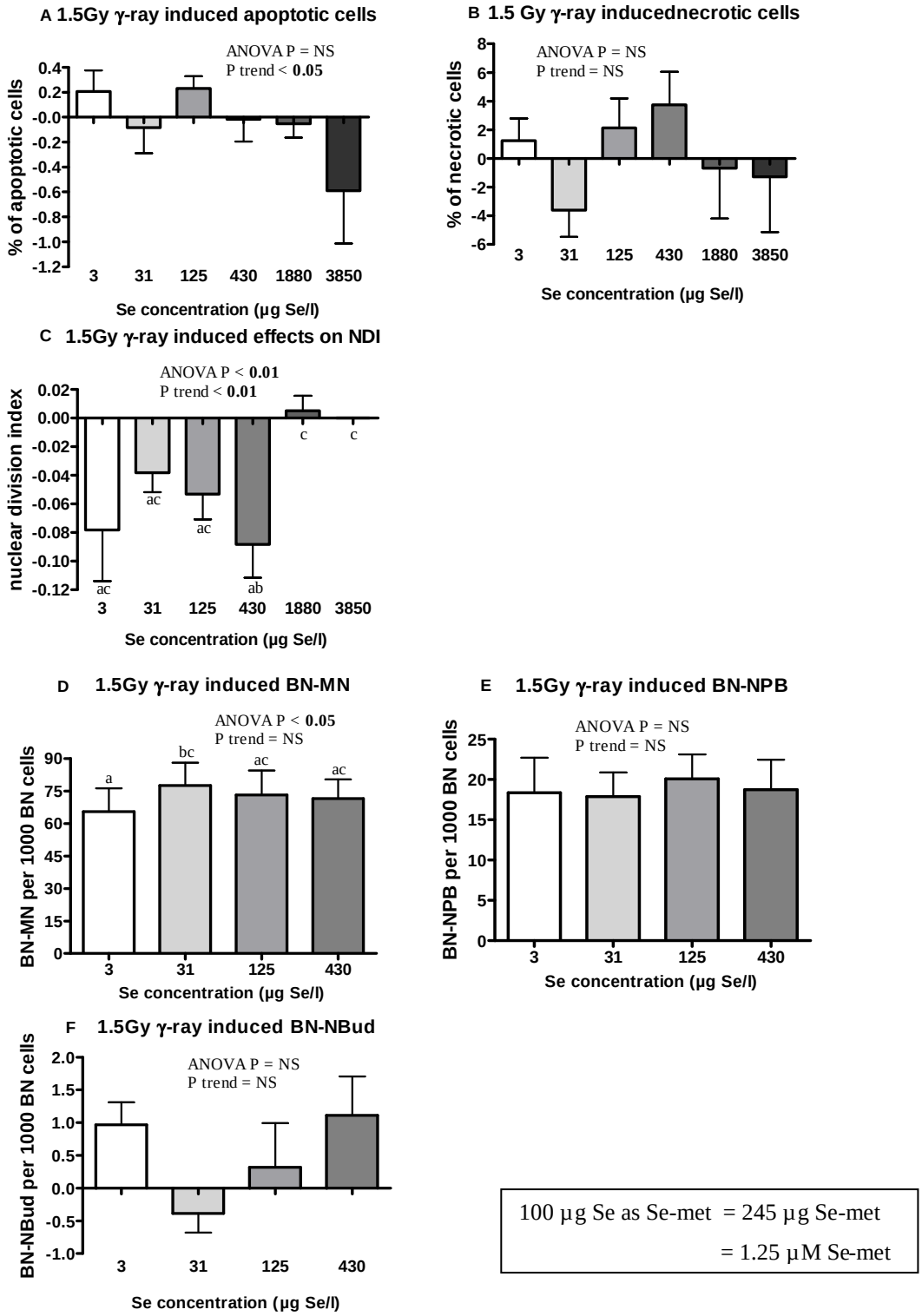


Figure 3-4 γ-irradiation induced cytotoxicity and genome damage endpoints scored using CBMN Cytome assay on cells exposed to γ irradiation on day 10 at increasing Se (as Se-met) concentrations: A. percentage of apoptotic cells; B. percentage of necrotic cells; C. nuclear division index; D. number of binucleated cells with micronuclei (BN-MN); E. number of binucleated cells with nucleoplasmic bridges (BN-NPB); F. number of binucleated cells with nuclear buds (BN-NBud). Groups not sharing the same letter are significantly different to each other ($P<0.05$). Results represent γ-ray induced values measured by subtracting values for non-irradiated controls from those of irradiated cultures. Results are shown as mean ± SE for cultures from six different subjects.

3.5 Discussion

The findings of this study suggest that Se-met *in vitro* (1) is cytotoxic at high concentrations (1880 µg Se/l or above); (2) may improve genome stability in lymphocytes at concentrations at the higher end of the physiological range (125-430 µg Se/l) and (3) has no protective effect against gamma radiation-induced genome damage.

Although Se-met is an organic dietary form of Se, our *in vitro* studies suggest it is cytotoxic to human lymphocytes at supra-physiological concentrations (≥ 1880 µg Se/l, 23.5 µM). Se-met cytotoxicity in human and murine cells *in vitro* varies depending on the cell type and culture conditions (Kajander et al., 1990; Weiller et al., 2004). This was indicated by markedly increased apoptotic and necrotic events and being highly cytostatic. In the Wil2-NS human B-lymphocyte cell line, Se-met caused 50% growth inhibition at a concentration of 6.2 mg Se/l (78 µM Se-met) over three days of culture (Kajander et al., 1990). However, normal human keratinocytes incubated with 25.3 mg Se/l (316.6 µM Se-met) Se-met for 24 hours did not show any effect on cell viability (Shen et al., 2001). When seleno-D,L-methionine, a form half as cytotoxic as Se-met (Schrauzer, 2000), was employed, 50% cell growth inhibitory effect was demonstrated at a concentration of 104 mg Se/l (1.3 mM Se-met) in 7-day normal human diploid fibrolasts cultures (Redman et al., 1998). In several human cancer cell lines, Se-met showed marked cytotoxicity at concentrations 3.2-12.8 mg Se/l (40-160 µM Se-met) (Kajander et al., 1990; Redman et al., 1998). However, a few studies showed that Se-met was essentially nontoxic to human liver and colon carcinoma cell lines up to 80-800 mg Se/l (1-10 mM Se-met) (Schwartz and Kmiec, 2007; Weiller et al., 2004). Se-met is known to be less cytotoxic than inorganic Se, such as selenite. It has been reported that selenite at concentrations of 480-800 µg Se/l (6-10 µM selenite) is cytotoxic to human lymphocytes causing increased frequency of micronuclei and inhibited cell division (Abul-Hassan et al., 2004; Berces et al., 1993; Biswas et al., 2000). This may be due to increased oxidative stress and DNA strand breaks in cells induced by selenite (Biswas et al., 2000; Kim et al., 2004; Zhou et al., 2003) (Takahashi et al., 2005).

The mechanisms by which Se-met exerts its cytotoxicity are not fully understood. Under physiological conditions, Se-met can be incorporated into proteins in place of methionine (Smith and Thompson, 1998). Excessive Se-met in proteins may alter their essential biological functions by changing the redox status of enzymes, interacting with thiols and zinc metabolism (Jacob et al., 2003) with consequent damage to living cells.

This may partly explain the cytotoxicity effect of Se-met at the two highest concentrations tested in this study, where 50% and 100% of methionine was provided as Se-met. Se-met can be metabolized to selenocysteine (Burk, 1991; Zhou et al., 2000), which is known to be highly reactive and several times more cytotoxic than Se-met (Kajander et al., 1990). At elevated cellular concentrations, selenocysteine can be randomly incorporated into proteins replacing cysteine (Muller et al., 1994) and might also contribute to Se cytotoxicity. It has also been suggested that the cytotoxicity of Se-met is superoxide mediated (Muller et al., 1994; Zhao et al., 2006). Se-met metabolites, hydrogen selenide and methyl selenol, can generate superoxide in the presence of methioninase and glutathione, resulting in overt consumption of intracellular reduced glutathione, increased oxidative stress and oxidative DNA damage, which lead to cell cycle arrest and apoptosis (Spallholz et al., 2004; Tapiero et al., 2003; Tarze et al., 2007). However, Se-met is more likely to be degraded through the selenocysteine – hydrogen selenide – methylselenol pathway when methionine supply is sufficient (Daniels, 1996).

The L form of Se-met is a suitable substrate in S-adenosylselenomethionine (SeAM) synthesis and competes with methionine for the same methionine adenosyltransferase reaction in the living cells (Kajander et al., 1990). It has been shown that SeAM produced from Se-met can be decarboxylated and functions effectively as aminopropyl group donor in polyamine synthesis in K562 cells (Human erythromyeloblastoid leukemia cell line) (Kajander et al., 1990; Porter et al., 1984). Se-met up to 800 µg Se/l (10 µM Se-met) could replace methionine and support the growth of several cell lines in absence of methionine when cell concentration is low, i.e. 0.5 x 10^5/ml (Kajander et al., 1991). It is known that polyamines are essential for multiple cell functions including cell proliferation and chromatid/DNA stability (Mamont et al., 1976; Minton et al., 1990; Pohjanpelto et al., 1985). Polyamine biosynthesis was not affected in L1210 cells cultured 48 hours with 10.4 mg Se/l (130 µM Se-met) of Se-met added in the medium (Porter et al., 1984). In contrast, a study in human A549 and HT29 cells suggested that induction in apoptosis and perturbation in the cell cycle in Se-met supplemented (5.2 mg Se/l or 65 µM Se-met) culture medium may be due to depleted polyamine levels, as evident by the administration of spermine, which prevented Se-met induced apoptosis (Redman et al., 1997). The efficiency of polyamine biosynthesis may vary in these transformed cell lines. Nevertheless, all these studies showed that increased Se-met concentration in culture medium with or without decreasing the concentration of methionine induced a profound decrease in the S-adenosylmethionine (SAM) pool

(Kajander et al., 1991; Kajander et al., 1990; Porter et al., 1984; Redman et al., 1997). SAM is utilized in almost 100 reactions which are of vital importance, such as in glutathione synthesis. Therefore, a decrease in the SAM pool may contribute to Se-met induced cytotoxicity. This is supported by the fact that Se-met cytotoxicity increased by 1.5 to 4-fold in various cell lines when methionine concentration in culture medium was as low as 10 µM (Kajander et al., 1990). Although SeAM could replace SAM in the transmethylation reaction, altered SeAM/SAM ratio may still interfere with the methylation process performed by various methyl transferases. It has been shown that RNA synthesis, which is highly modified by methylation, decreased significantly after 48 hour in R1.1 cells cultured with addition of 12.5 mg Se/l (156 µM Se-met) Se-met (Kajander et al., 1990). It has been reported that DNA cytosine methyltransferase activity was inhibited by selenium compounds benzyl selenocyanate and 1.4-phenylenebis(methylene)-selenocyanate, as well as sodium selenite in dose-dependent manner in HCT116 human colon carcinoma cells (Fiala et al., 1998). In our study, total methionine concentration in culture medium was kept constant, while Se-met concentration increased, L-methionine concentration decreased. Therefore, the effect observed in our study may also reflect the impact of decreased methionine and a reduction in SAM concentration, if the latter occurred.

The present study shows that increasing Se concentration from 3 to 430 µg Se/l in the form of Se-met reduced genome damage in lymphocytes as indicated by the significant trend of reduction in the frequency of BN-NPB and the frequency of BN-NBud without increasing the frequency of MN. BN-NPB, is a biomarker of DNA mis-repair, chromosome rearrangement or telomere end-fusion. BN-NBud is a biomarker of gene amplification that may be generated via bridge-breakage-fusion cycles initiated by NPBs from telomere end fusions (Fenech and Crott, 2002). The reduced frequency of NPBs and NBuds in cultured lymphocytes with increasing Se concentration might be a result of enhanced telomere stability. Recent studies have shown that supplementing culture medium with physiological doses of Se (40 and 200 µg Se/l) as sodium selenite increased telomerase activity and extended telomere length in human L-02 hepatocytes in a dose-response manner when compared to the Se-deficient control (Liu et al., 2003; Liu et al., 2004). Therefore, increasing Se to 400 µg Se/l in the form of Se-met might also limit the rate of telomere shortening and the formation of dicentric chromosomes due to telomere end fusion (caused by telomere shortening), which are expressed as NPBs (Fenech, 2006). Se-met could possibly reduce genome instability by modulating DNA repair at relatively high concentrations. Se-met at 16 µg Se/l (200 nM Se-met) has

no effect on excision repair in primary human keratinocytes (Rafferty et al., 2003). However, pre-treatment with 800 µg Se/l (10 µM Se-met) Se-met for 15 hours can elevate DNA repair response (Seo et al., 2002b) in cells with normal functioning p53 (Fischer et al., 2007; Seo et al., 2002a; Traynor et al., 2006). It has been shown that Se-met at 5 µM increased *ATR* and *CHK2* gene expression in human thyroid cell line (Kennedy et al., 2004). *ATR* and *CHK2* gene products are important cell cycle checkpoint proteins in genome integrity maintenance, which could prevent cells from going through mitosis until the damage is repaired. A recent study suggested that the frequencies of BN-NPB and BN-NBud are predictive of lung cancer risk (El-Zein et al., 2006). Therefore, Se-met at the higher end of physiological concentration range may reduce susceptibility to BN-NPB and BN-NBud formation and possibly reduce lung cancer risk in populations with low Se status, such as smokers (Reid et al., 2002; Zhuo et al., 2004). However, further studies are needed to prove this potential protective effect of Se-met *in vivo*.

We did not observe a beneficial effect of Se-met on MN-BN frequency. This may be due to MN being generated as a result of chromosome breaks as well as lagging whole chromosomes. Because oxidative stress increases MN formation in human lymphocytes (Greenrod and Fenech, 2003; Umegaki and Fenech, 2000) and Se supplementation is expected to increase activity of selenoenzymes involved in antioxidant response (Lewin et al., 2002), it is reasonable to expect a reduction in MN caused by oxidative stress with increasing Se-met in the physiological range. On the other hand, as explained above some studies have shown a reduction in SAM production with increasing Se concentration *in vitro*. SAM is required for maintenance of methylation of repeat sequences at the centromere which is essential for centromere structural integrity and functionality (Fenech, 2001). It has been shown that hypomethylation of DNA induced by treatment with 5-azacytridine increases formation of MN (Guttenbach and Schmid, 1994). It is therefore also plausible that Se-met-induced DNA hypomethylation might increase MN formation. The two potential mechanisms described above which have opposing effects on MN formation may explain the apparent null effect on MN formation and could only be resolved in future studies by analysing MN for presence or absence of centromeric DNA to distinguish between MN originating from chromosome breaks or chromosome loss.

Another possible explanation for a null effect of Se-Met on MN frequency in our study is that cells with induced MN in the earlier stages of the culture period were at a disadvantage to further propagate. However, our previous studies with folic acid in 9

day cultures consistently showed a dose-related reduction in MN frequency which is due to the continuous generation of MN in those cells that can divide (Crott et al., 2001). Even if cells with MN were at a disadvantage to divide those that could would still be susceptible to chromosome damage events if micronutrient status was sub-optimal. Other studies suggest a protective effect of Se on radiation-induced MN formation may be more evident in cell lines that do not adequately express glutathione peroxidase (Baliga et al., 2008; Baliga et al., 2007). Further studies with lymphocytes defective in this antioxidant enzyme may be more informative to test whether our observations are also applicable to such genetic sub-types.

The observation in this study that Se-met within the physiological range had no impact on DNA damage and cytotoxicity induced by ionising radiation is somewhat unexpected given the known role of certain selenoenzymes such as glutathione peroxidase on attenuating the effect of reactive oxygen species. However, these observations are consistent with those of Shin *et al*. (Shin et al., 2007) and Sandstrom *et al*. (Sandstrom et al., 1989), which showed no impact of Se-met or selenite on DNA damage induced by ionising radiation and imply that increased activity of antioxidant selenoenzymes may have no role to play in modulating the effects of ionising radiation in normal human cells.

In conclusion, Se-met may improve genome stability in lymphocytes at Se concentrations between 100 and 430 µg Se/l. However, Se-met can be cytotoxic at higher doses. Therefore, a cautious approach to Se supplementation is required to ensure that optimal genome stability is achieved without cytotoxic effects.

3.6 References

ABUL-HASSAN, K. S., LEHNERT, B. E., GUANT, L. & WALMSLEY, R. (2004) Abnormal DNA repair in selenium-treated human cells. *Mutation Research,* 565**,** 45-51.

AMES, B. N., SHIGENAGA, M. K. & HAGEN, T. M. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 7915-7922.

ARTHUR, J. (2000) The glutathione peroxidases. *CMLS Cellular and Molecular Life Sciences,* 57**,** 1825-1835.

BALIGA, M. S., DIWADKAR-NAVSARIWALA, V., KOH, T., FAYAD, R., FANTUZZI, G. & DIAMOND, A. M. (2008) Selenoprotein deficiency enhances radiation-induced micronuclei formation. *Molecular Nutrition and Food Research,* 52**,** 1300-1304.

BALIGA, M. S., WANG, H., ZHUO, P., SCHWARTZ, J. L. & DIAMOND, A. M. (2007) Selenium and GPx-1 overexpression protect mammalian cells against UV-induced DNA damage. *Biological Trace Element Research,* 115**,** 227-242.

BERCES, J., OTOS, M., SZIRMAI, S., CRANE-URUENA, C. & KOTELES, G. J. (1993) Using the micronucleus assay to detect genotoxic effects of metal ions. *Environmental Health Perspectives,* 101 Suppl 3**,** 11-13.

BISWAS, S., TALUKDER, G. & SHARMA, A. (2000) Chromosome damage induced by selenium salts in human peripheral lymphocytes. *Toxicology In Vitro,* 14**,** 405-408.

BONASSI, S., ZNAOR, A., CEPPI, M., LANDO, C., CHANG, W. P., HOLLAND, N., KIRSCH-VOLDERS, M., ZEIGER, E., BAN, S., BARALE, R., BIGATTI, M. P., BOLOGNESI, C., CEBULSKA-WASILEWSKA, A., FABIANOVA, E., FUCIC, A., HAGMAR, L., JOKSIC, G., MARTELLI, A., MIGLIORE, L., MIRKOVA, E., SCARFI, M. R., ZIJNO, A., NORPPA, H. & FENECH, M. (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis,* 28**,** 625-631.

BURK, R. F. (1991) Molecular biology of selenium with implications for its metabolism. *FASEB Journal,* 5**,** 2274-2279.

CLARK, L., DALKIN, B., KRONGRAD, A., COMBS, G., JR., TURNBULL, B., SLATE, E., WITHERINGTON, R., HERLONG, J., JANOSKO, E., CARPENTER, D., BOROSSO, C., FALK, S. & ROUNDER, J. (1998) Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *British Journal of Urology,* 81**,** 730-734.

COMBS, G., JR. (2001a) Selenium in global food systems. *British Journal of Nutrition,* 85**,** 517-547.

COMBS, G., JR. (2005) Current evidence and research needs to support a health claim for selenium and cancer prevention. *Journal of Nutrition,* 135**,** 343-347.

COMBS, G. F., JR. (2001b) Impact of selenium and cancer-prevention findings on the nutrition-health paradigm. *Nutrition and Cancer,* 40**,** 6-11.

CROTT, J. W., MASHIYAMA, S. T., AMES, B. N. & FENECH, M. (2001) The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 1089-1096.

DANIELS, L. (1996) Selenium metabolism and bioavailability. *Biological Trace Element Research,* 54**,** 185-199.

DUFFIELD-LILLICO, A. J., DALKIN, B. L., REID, M. E., TURNBULL, B. W., SLATE, E. H., JACOBS, E. T., MARSHALL, J. R. & CLARK, L. C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU International,* 91**,** 608-612.

EL-ZEIN, R. A., SCHABATH, M. B., ETZEL, C. J., LOPEZ, M. S., FRANKLIN, J. D. & SPITZ, M. R. (2006) Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Research,* 66**,** 6449-6456.

ETMINAN, M., FITZGERALD, J. M., GLEAVE, M. & CHAMBERS, K. (2005) Intake of selenium in the prevention of prostate cancer: a systematic review and meta-analysis. *Cancer Causes and Control,* 16**,** 1125-1131.

FENECH, M. (2001) The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutation Research,* 475**,** 57-67.

FENECH, M. (2006) Cytokinesis-block micronucleus assay evolves into a “cytome” assay of chromosomal instability, mitotic dysfunction and cell death. *Mutation Research,* 600**,** 58-66.

FENECH, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nature Protocol,* 2**,** 1084-1104.

FENECH, M. (2008) Genome health nutrigenomics and nutrigenetics--diagnosis and nutritional treatment of genome damage on an individual basis. *Food and Chemical Toxicology,* 46**,** 1365-1370.

FENECH, M. & CROTT, J. W. (2002) Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research,* 504**,** 131-136.

FIALA, E., STARETZ, M., PANDYA, G., EL-BAYOUMY, K. & HAMILTON, S. (1998) Inhibition of DNA cytosine methyltransferase by chemopreventive selenium compounds, determined by an improved assay for DNA cytosine methyltransferase and DNA cytosine methylation. *Carcinogenesis,* 19**,** 597-604.

FISCHER, J. L., MIHELC, E. M., POLLOK, K. E. & SMITH, M. L. (2007) Chemotherapeutic selectivity conferred by selenium: a role for p53-dependent DNA repair. *Molecular Cancer Therapeutics,* 6**,** 355-361.

GREENROD, W. & FENECH, M. (2003) The principal phenolic and alcoholic components of wine protect human lymphocytes against hydrogen peroxide- and ionizing radiation-induced DNA damage in vitro. *Mutagenesis,* 18**,** 119-126.

GUTTENBACH, M. & SCHMID, M. (1994) Exclusion of specific human chromosomes into micronuclei by 5-azacytidine treatment of lymphocyte cultures. *Experimental Cell Research,* 211**,** 127-132.

HATFIELD, D. L., CARLSON, B. A., XU, X. M., MIX, H. & GLADYSHEV, V. N. (2006) Selenocysteine incorporation machinery and the role of selenoproteins in development and health. *Progress in Nucleic Acid Research and Molecular Biology,* 81**,** 97-142.

HENDERSON, L., JONES, E., BROOKS, T., CHETELAT, A., CILIUTTI, P., FREEMANTLE, M., HOWARD, C. A., MACKAY, J., PHILLIPS, B., RILEY, S., ROBERTS, C., WOTTON, A. K. & VAN DE WAART, E. J. (1997) Industrial Genotoxicology Group collaborative trial to investigate cell cycle parameters in human lymphocyte cytogenetics studies. *Mutagenesis,* 12**,** 163-167.

IP, C., BIRRINGER, M., BLOCK, E., KOTREBAI, M., TYSON, J. F., UDEN, P. C. & LISK, D. J. (2000) Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. *Journal of Agricultural and Food Chemistry,* 48**,** 4452.

JACOB, C., GILES, G. I., GILES, N. M. & SIES, H. (2003) Sulfur and selenium: the role of oxidation state in protein structure and function. *Angewandte Chemie. International Ed. In English,* 42**,** 4742-4758.

KAJANDER, E. O., HARVIMA, R. J., ELORANTA, T. O., MARTIKAINEN, H., KANTOLA, M., KARENLAMPI, S. O. & AKERMAN, K. (1991) Metabolism, cellular actions, and cytotoxicity of selenomethionine in cultured cells. *Biological Trace Element Research,* 28**,** 57-68.

KAJANDER, E. O., HARVIMA, R. J., KAUPPINEN, L., AKERMAN, K. K., MARTIKAINEN, H., PAJULA, R. L. & KARENLAMPI, S. O. (1990) Effects of selenomethionine on cell growth and on S-adenosylmethionine metabolism in cultured malignant cells. *Biochemical Journal,* 267**,** 767-774.

KENNEDY, A., WARE, J., GUAN, J., DONAHUE, J., BIAGLOW, J., ZHOU, Z., STEWART, J., VAZQUEZ, M. & WAN, X. (2004) Selenomethionine protects against adverse biological effects induced by space radiation. *Free Radical Biology and Medicine,* 36**,** 259-266.

KIM, Y. S., JHON, D. Y. & LEE, K. Y. (2004) Involvement of ROS and JNK1 in selenite-induced apoptosis in Chang liver cells. *Experimental and Molecular Medicine,* 36**,** 157-164.

KIMURA, M., UMEGAKI, K., HIGUCHI, M., THOMAS, P. & FENECH, M. (2004) Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *Journal of Nutrition,* 134**,** 48-56.

LEWIN, M. H., ARTHUR, J. R., RIEMERSMA, R. A., NICOL, F., WALKER, S. W., MILLAR, E. M., HOWIE, A. F. & BECKETT, G. J. (2002) Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell line EAhy926 from damage by lipid hydroperoxides. *Biochimica et Biophysica Acta,* 1593**,** 85-92.

LIU, Q., WANG, H., HU, D., DING, C., XIAO, H., XU, H., SHU, B. & XU, S. (2003) Effects of sodium selenite on telomerase activity and telomere length. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai),* 35**,** 1117-1122.

LIU, Q., WANG, H., HU, D., DING, C., XU, H. & TAO, D. (2004) Effects of trace elements on the telomere lengths of hepatocytes L-02 and hepatoma cells SMMC-7721. *Biological Trace Element Research,* 100**,** 215-227.

MAMONT, P. S., BOHLEN, P., MCCANN, P. P., BEY, P., SCHUBER, F. & TARDIF, C. (1976) Alpha-methyl ornithine, a potent competitive inhibitor of ornithine decarboxylase, blocks proliferation of rat hepatoma cells in culture. *Proceedings of the National Academy of Sciences of the United States of America,* 73**,** 1626-1630.

MINTON, K. W., TABOR, H. & TABOR, C. W. (1990) Paraquat toxicity is increased in Escherichia coli defective in the synthesis of polyamines. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 2851-2855.

MULLER, S., SENN, H., GSELL, B., VETTER, W., BARON, C. & BOCK, A. (1994) The formation of diselenide bridges in proteins by incorporation of selenocysteine residues: biosynthesis and characterization of (Se)2-thioredoxin. *Biochemistry,* 33**,** 3404-3412.

PAPP, L. V., LU, J., HOLMGREN, A. & KHANNA, K. K. (2007) From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxidants and Redox Signalling,* 9**,** 775-806.

POHJANPELTO, P., HOLTTA, E., JANNE, O. A., KNUUTILA, S. & ALITALO, K. (1985) Amplification of ornithine decarboxylase gene in response to polyamine deprivation in Chinese hamster ovary cells. *Journal of Biological Chemistry,* 260**,** 8532-8537.

PORTER, C. W., SUFRIN, J. R. & KEITH, D. D. (1984) Growth inhibition by methionine analog inhibitors of S-adenosylmethionine biosynthesis in the absence of polyamine depletion. *Biochemical and Biophysical Research Communications,* 122**,** 350-357.

RAFFERTY, T., GREEN, M., LOWE, J., ARLETT, C., HUNTER, J., BECKETT, G. & MCKENZIE, R. (2003) Effects of selenium compounds on induction of DNA damage by broadband ultraviolet radiation in human keratinocytes. *British Journal of Dermatology,* 148**,** 1001-1009.

REDMAN, C., SCOTT, J. A., BAINES, A. T., BASYE, J. L., CLARK, L. C., CALLEY, C., ROE, D., PAYNE, C. M. & NELSON, M. A. (1998) Inhibitory effect of selenomethionine on the growth of three selected human tumor cell lines. *Cancer Letters,* 125**,** 103-110.

REDMAN, C., XU, M. J., PENG, Y. M., SCOTT, J. A., PAYNE, C., CLARK, L. C. & NELSON, M. A. (1997) Involvement of polyamines in selenomethionine induced apoptosis and mitotic alterations in human tumor cells. *Carcinogenesis,* 18**,** 1195-1202.

REEVES, P. G., GREGOIRE, B. R., GARVIN, D. F., HARELAND, G. A., LINDLAUF, J. E., JOHNSON, L. K. & FINLEY, J. W. (2007) Determination of selenium bioavailability from wheat mill fractions in rats by using the slope-

ratio assay and a modified torula yeast-based diet. *Journal of Agricultural and Food Chemistry,* 55**,** 516-522.

REID, M. E., DUFFIELD-LILLICO, A. J., GARLAND, L., TURNBULL, B. W., CLARK, L. C. & MARSHALL, J. R. (2002) Selenium supplementation and lung cancer incidence: an update of the nutritional prevention of cancer trial. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 1285-1291.

SANDSTROM, B. E., GRANKVIST, K. & MARKLUND, S. L. (1989) Selenite-induced increase in glutathione peroxidase activity protects human cells from hydrogen peroxide-induced DNA damage, but not from damage inflicted by ionizing radiation. *International Journal of Radiation Biology,* 56**,** 837-841.

SCHRAUZER, G. (2000) Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *Journal of Nutrition,* 130**,** 1653-1656.

SCHWARTZ, T. R. & KMIEC, E. B. (2007) Reduction of gene repair by selenomethionine with the use of single-stranded oligonucleotides. *BMC Molecular Biology,* 8**,** 7.

SEO, Y., KELLEY, M. & SMITH, M. (2002a) Selenomethionine regulation of p53 by a ref1-dependent redox mechanism. *Proc Natl Acad Sci U. S. A.,* 99**,** 14548-14553.

SEO, Y. R., SWEENEY, C. & SMITH, M. L. (2002b) Selenomethionine induction of DNA repair response in human fibroblasts. *Oncogene,* 21**,** 3663-3669.

SHEN, C., SONG, W. & PENCE, B. (2001) Interactions of selenium compounds with other antioxidants in DNA damage and apoptosis in human normal keratinocytes. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 385-390.

SHIN, S. H., YOON, M. J., KIM, M., KIM, J. I., LEE, S. J., LEE, Y. S. & BAE, S. (2007) Enhanced lung cancer cell killing by the combination of selenium and ionizing radiation. *Oncology Reports,* 17**,** 209-216.

SMITH, J. L. & THOMPSON, A. (1998) Reactivity of selenomethionine--dents in the magic bullet? *Structure,* 6**,** 815-819.

SPALLHOLZ, J., PALACE, V. & REID, T. (2004) Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an in vitro chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoamino acids. *Biochemical Pharmacology,* 67**,** 547-554.

TAKAHASHI, M., SATO, T., SHINOHARA, F., ECHIGO, S. & RIKIISHI, H. (2005) Possible role of glutathione in mitochondrial apoptosis of human oral squamous cell carcinoma caused by inorganic selenium compounds. *International Journal of Oncology,* 27**,** 489-495.

TAPIERO, H., TOWNSEND, D. & TEW, K. (2003) The antioxidant role of selenium and seleno-compounds. *Biomedicine and Pharmacotherapy,* 57**,** 134-144.

TARZE, A., DAUPLAIS, M., GRIGORAS, I., LAZARD, M., HA-DUONG, N. T., BARBIER, F., BLANQUET, S. & PLATEAU, P. (2007) Extracellular production of hydrogen selenide accounts for thiol-assisted toxicity of selenite against Saccharomyces cerevisiae. *Journal of Biological Chemistry,* 282**,** 8759-8767.

TRAYNOR, N. J., MCKENZIE, R. C., BECKETT, G. J. & GIBBS, N. K. (2006) Selenomethionine inhibits ultraviolet radiation-induced p53 transactivation. *Photodermatology, Photoimmunology and Photomedicine,* 22**,** 297-303.

UMEGAKI, K. & FENECH, M. (2000) Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils. *Mutagenesis,* 15**,** 261-269.

WANG, X., THOMAS, P., XUE, J. & FENECH, M. (2004) Folate deficiency induces aneuploidy in human lymphocytes in vitro-evidence using cytokinesis-blocked

cells and probes specific for chromosomes 17 and 21. *Mutation Research,* 551**,** 167-180.

WEILLER, M., LATTA, M., KRESSE, M., LUCAS, R. & WENDEL, A. (2004) Toxicity of nutritionally available selenium compounds in primary and transformed hepatocytes. *Toxicology,* 201**,** 21-30.

ZARCINAS, B., CARTWRIGHT, B. & SPOUNCER, L. (1987) Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. *Communications in Soil Science and Plant Analysis,* 18**,** 131-146.

ZARCINAS, B., MCLAUGHLIN, M. & SMART, M. (1996) The effect of acid digestion technique on the performance of nebulization systems used in inductively coupled plasma spectrometry. *Communications in Soil Science and Plant Analysis,* 27**,** 1331-1354.

ZHAO, R., DOMANN, F. E. & ZHONG, W. (2006) Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells. *Molecular Cancer Therapeutics,* 5**,** 3275-3284.

ZHOU, N., XIAO, H., LI, T., NUR, E., A, K. & LIU, L. (2003) DNA damage-mediated apoptosis induced by selenium compounds. *Journal of Biological Chemistry,* 278**,** 29532-29537.

ZHOU, Z., SMITH, A. & MATTHEWS, R. (2000) L-selenohomocysteine: one-step synthesis from L-selenomethionine and kinetic analysis as substrate for methionine synthases. *Bioorganic & Medicinal Chemistry Letters,* 10**,** 2471-2475.

ZHUO, H., SMITH, A. H. & STEINMAUS, C. (2004) Selenium and lung cancer: a quantitative analysis of heterogeneity in the current epidemiological literature. *Cancer Epidemiology, Biomarkers and Prevention,* 13**,** 771-778.

4. The Effect of Se (as Seleno-L-methionine) Concentration on DNA Damage in Human Lymphocytes as Measured Using the Comet Assay

4.1 Summary

A supra-nutritional intake of selenium (Se) may be required for reduction of cancer risk, but an excessively high dose could be cytotoxic. Accumulated genome damage is a fundamental cause of cancer development. Therefore, the effect of seleno-L-methionine (Se-met), one of the major dietary forms of Se, on DNA damage was investigated to determine the bio-efficacy and upper safety limit of this micronutrient. Peripheral blood lymphocytes were isolated from six volunteers and cultured with medium supplemented with Se-met in a series of Se concentrations (3, 31, 125, 430, 1880 and 3850 µg Se/l) while keeping methionine concentration constant at 50 µM. Baseline genome stability of lymphocytes and the extent of DNA damage induced by 7.5 µM hydrogen peroxide (H_2O_2) were investigated using the alkaline Comet assay after 9 days of culture in 96-microwell plates. Glycosylase (Fpg and Endo III) treatment was also employed to allow the oxidized purines and pyriminidines to be expressed as single strand breaks and then determined using the alkaline Comet assay. High Se concentrations (1880 and 3850 µg Se/l) inhibited cell growth as indicated by low cell concentration ($P<0.0001$) and extensive DNA damage ($P<0.0001$) compared to non-cytotoxic concentrations of Se. Selenium concentration ($\leq$ 430 µg Se/l) had no effect on baseline DNA damage or on H_2O_2-induced oxidative DNA damage, including glycosylase-sensitive sites. Furthermore, increasing Se concentration showed a significant trend for increase in oxidative DNA damage (P trend <0.05) in lymphocytes. In conclusion, Se-met is cytotoxic at high dose (1880 µg Se/l and 3850 µg/l). Increased Se concentration up to 430 µg/l may increase cellular oxidative stress. Therefore, a cautious approach to Se supplementation is required to ensure that optimal genome stability is achieved in long term cell cultures without undesired effects. Se-met is not effective in diminishing DNA damage caused by H_2O_2 in vitro.

4.2 Introduction

Recent studies have suggested that supra-nutritional intake of selenium (Se) may have beneficial role in human health, such as reducing cancer risks (Combs, 2005; Papp et al., 2007). As an essential micronutrient, Se exerts its biological functions through more than 20 selenoproteins in body, such as glutathione peroxidases (GPxs) and thioredoxin reductases (TRxs) (Papp et al., 2007). Accumulation of genome damage or instability is considered one of the fundamental causes of cancers (Fenech, 2002). Oxidative damage to DNA is regarded as an important step in carcinogenesis, therefore, measurement of these types of DNA lesion may be indicative of effects on cancer risk (Keck and Finley, 2006; Loft et al., 2008). Se may protect DNA from oxidative damage by improving function of GPxs as free radical scavengers. However, the role of Se in genome stability is largely unknown.

The Comet assay provides a sensitive and effective way for evaluating DNA damage at the single-cell level. Therefore it has been extensively used as a genotoxicity test for investigation of the effects of antioxidants on oxidative-stress related oxidative DNA damage in various cell types as well as for in vivo biomonitoring (Møller and Loft, 2002). The assay measures unrepaired DNA single-strand breaks, transient repair sites and abasic sites. Oxidized DNA bases can also be expressed and visualized as single strand breaks in the alkaline Comet assay by including an incubation step with purified repair enzyme that recognizes a specific type of base damage (Collins, 2004; Collins, 2005). The formamidopyrimidine-DNA glycosylase (Fpg) and the endonuclease III (Endo III) are two of the glycosylases commonly used in the Comet assay to recognize oxidized purines and pyrimidines, respectively. As the first step of base excision repair mechanism, Fpg or Endo III detects and removes oxidised bases, such as 7, 8-dihydro-8-oxoguanine (8-oxoguanine), to produce abasic sites, which can be recognized by other DNA repair enzymes (Boiteux et al., 1992; Tchou et al., 1991). During the alkaline Comet assay, DNA unwinding allows these transient repair sites or abasic sites to be expressed as single strand breaks, and are thus referred to as glycosylase-sensitive sites. Measuring a combination of endogenous single strand breaks and glycosylase-sensitive sites provides a more comprehensive assessment of cellular oxidative stress-induced DNA damage (Cemeli et al., 2008). Increased DNA damage and cell death measured by the Comet assay has been shown to be associated with aging in humans (Piperakis et al., 2009).

Se-met is one of the major forms of dietary Se that occurs naturally in many plant foods, such as wheat. However, the upper and lower safety limits of Se-met are not well defined. The present study aimed to test the genotoxicity and cytoxicity effect of Se-met at a range of physiologically relevant concentrations using a primary lymphocyte long-term culture protocol to 1) verify the safe dose-range and bio-efficacy of Se-met; 2) determine the optimal concentration of Se-met for reduction of oxidative DNA damage and 3) determine the optimal concentration of Se-met for improving resistance to hydrogen peroxide (H_2O_2) induced oxidative DNA damage.

4.3 Study design and methods

4.3.1 Recruitment

Six male volunteers were recruited from staff members of CSIRO Human Nutrition or University of Adelaide after giving informed consent. Inclusion criteria were: 1) healthy males aged 18-65 yrs, 2) not supplementing with Se or having refrained from supplementation for 4 weeks if taking >2x recommended dietary intake of Se supplement and 3) not taking above the recommended daily intake (RDI) levels of folate, vitamin B_{12}, vitamin C per day on average or having refrained from supplementation for 1 week prior to giving blood. Ethics approval was obtained from the Human Research Ethics Committee of CSIRO Human Nutrition.

Volunteers were required to complete a standard general lifestyle questionnaire while attending the clinic, in which details of any supplements taken were recorded, such as product brand, active ingredients per tablet, dose and frequency of intake. In addition, the quantity and frequency of Brazil nut consumption was also determined because these nuts are usually rich in Se (Reeves et al., 2007).

4.3.2 Collection of blood samples and determination of Se

For each volunteer, 18 ml of whole blood was collected by venipuncture into lithium-heparin vacutainer tubes (Vacuette, Austria). Blood was taken between 8 am – 11 am after an overnight fast and with only a light breakfast. (i.e. a slice of toast without butter and one cup of coffee/tea with low-fat milk). Two ml of plasma was obtained from each sample by centrifuging 5 ml of whole blood at 3000 rpm for 20 min. Plasma Se level was measured at Waite Analytical Services (WAS) using inductively coupled plasma

mass spectrometry (ICP-MS) after acid digestion (Zarcinas et al., 1987; Zarcinas et al., 1996).

4.3.3 Lymphocyte isolation

12 ml of the blood sample was diluted with an equal volume of Hanks balanced salt solution (HBSS, Thermo, US) and mixed. To isolate lymphocytes, 8 ml Ficol Paque (Amersham Pharmacia Biotech, Sweden) was added to a 50 ml Falcon tube and 24 ml of diluted blood was gently overlaid. The tubes were centrifuged at 400 g for 30 min at 22^{o}C. The cells were collected from the fluffy layer and washed with 3 volumes of HBSS, then centrifuged at 180 g for 10 min at room temperature. The cell pellet in the Falcon tube (BD, Australia) was resuspended in 18 ml of HBSS and 3 ml of the cell suspension was transferred into each of 6x TV-10 tubes (Techno Plas, Australia). The cells were washed once more in HBSS and the cell pellet was resuspended in 1 ml of appropriate Se-supplemented culture medium containing 10% foetal bovine serum (FBS, Trace MultiCel, Australia). The concentration of cells was adjusted to 1 x 10^6 cells/ml, then further diluted to obtain 6 ml of 1.5 x 10^5 cells/ml suspension with appropriate culture medium according to the cell count results.

4.3.4 Cell counting using the Coulter Counter

Cell count was measured using a Coulter Counter (Model Z1, Beckman, Australia). The count of each sample was taken in triplicate and the mean value of samples containing cells measured. The mean of blank counts was subtracted from the mean cell count before calculating the cell concentration. Cell concentration (cells/ml) was determined by multiplying the value by 2000 (dilution factor 1000 and counting volume of 0.5 ml).

4.3.5 Culture medium

Se-met supplemented culture medium was prepared using Se-deficient RPMI-1640 (Sigma, Australia) medium deficient in L-methionine, L-cystine, L-glutamine and selenium, to which 10% FBS (Trace MultiCel, Australia) was added. Se concentration in FBS was 13 µg Se/l and therefore effectively only contributed 1.3 µg Se/l to the medium. L-glutamine (Sigma, Australia) and L-cystine (Sigma, Australia) were added to achieve final concentrations of 2.0 mM and 0.27 mM, respectively, to match with the components and concentrations in a standard RPMI-1640 recipe. The final 50 µM total methionine concentration (either L-methionine or seleno-L-methionine or a combination of both, Sigma, Australia) was chosen because it sustains cell division in vitro in long term cultures and is close to the physiological range in plasma in vivo (20-

30 µM) (Kimura et al., 2004). Using different seleno-L-methionine/methionine (without Se) ratios, it was possible to construct six different culture media that only differed in the content of Se and were otherwise identical in total methionine (Se-met plus L-methionine content) concentration. The intended and actual concentrations measured by ICP-MS (shown in brackets) were 1 (3), 120 (125), 480 (430), 1920 (1880) and 3950 (3850) µg Se/l. The highest concentration represented the situation for all of methionine being supplied as Se-met; i.e. 3950 µg Se/l Se is equivalent to 50 µM Se-met. For the purpose of this study, Se-met concentration is shown as µg Se/l to enable comparison with the conventional manner of expressing Se concentration *in vivo*. For conversion to µM values, the following equation is used: 100 µg Se as Se-met = 1.25 µM Se-met.

Calculations for preparing a total final volume of 117.6 ml culture medium with 6 different Se concentrations are specified in Table 4-1 and Table 4-2.

Table 4-1 Se concentrations in culture medium

Treatments	A	B	C	D	E	F
Intended final Se in medium (µg/l)	1	30	120	480	1920	3950
Actual measured Se in meidum (µg/l)	3	31	125	430	1880	3850
Intended final Se-met in medium (µM)	0	0.4	1.5	6.0	24.1	50
Intended final met in medium (µM)	50	49.6	48.5	44.0	25.9	0

Table 4-2 Preparation of culture medium with varying Se concentrations

Treatments	A	B	C	D	E	F
RPMI-1640 deficient medium * (ml)	95.3	95.3	95.3	95.3	95.3	95.3
FBS (10% final) (ml)	11.8	11.8	11.8	11.8	11.8	11.8
100 mM Sodium pyruvate solution ** (ml)	1.18	1.18	1.18	1.18	1.18	1.18
Pen/Strep antibiotic solution *** (ml)	1.18	1.18	1.18	1.18	1.18	1.18
29.2 mg/l stock L-glutamine solution (ml)	1.18	1.18	1.18	1.18	1.18	1.18
5.9 mg/l stock L-cystine.2HCl solution (ml)	1.18	1.18	1.18	1.18	1.18	1.18
298.4 µg/l stock L-methionine solution (µl)	2941	2920	2853	2585	1512	0
196.1 µg/l stock seleno-L-methionine solution (µl)	0	43	177	713	2859	5883
Extra RPMI-1640 to make up 117.6 ml (µl)	2941	2920	2853	2585	1512	0

* RPMI-1640 medium was deficient in L-glutamine, L-cystine and L-methionine – the stock solutions of these agents were prepared in the RPMI-1640 medium and sterilized using 0.22 µm filter.

** Sodium pyruvate (Sigma) was purchased as a sterile 100 mM solution in saline, final concentration in culture medium = 1 mM.

*** Penicillin/Streptomycin (Sigma) was purchased as a sterile solution of 5000 IU/ml Penicillin / 5000 µg/ml Streptomycin in tissue culture grade water, final concentration in culture medium 50 IU Penicillin / 50 µg/ml Streptomycin.

4.3.6 9-day lymphocyte culture in 96-microwell plate

4.3.6.1 9-day lymphocyte culture in 96-microwell plate (Figure 4-1)

Isolated lymphocytes were cultured in 96-microwell plates (NUNC flat bottom, sterile with lid, 200 µl per well) with appropriate seleno-L-methionine supplemented culture medium at a concentration of 1.5 x 10^5 cells/ml in 200 µl volume. Six ml of 1.5 x 10^5 cells/ml cell suspension was prepared with each appropriate culture medium in a multi-channel reservoir, then transferred to the wells of a microwell plate. In such cell suspension, phytohaemagglutinin (PHA, Abbott Murex #HA15 8E27, Australia) and Interleukin-2 (IL-2, Roche Diagnostics Cat# 799068, Australia) were added to stimulate cell division and growth using a final concentration of PHA of 30 µg/ml and 4 units of IL-2 per 200 µl culture volume in each well. The seeding concentration of lymphoyctes was determined in preliminary experiment aimed at optimising percentage of dividing cells identified as binucleated cells accumulated 24 hours following Cytochalasin-B (Cyto-B, Sigma, Australia) treatment on day 9. Time of adding PHA was recorded on day 0. Cells were cultured in a humidified incubator at 37°C with 5% CO_2 (Sanyo MCO-17 A1C, Japan) for 6 days. On day 7, half of the culture medium was removed carefully without disturbing the cells and replaced with fresh culture medium with IL-2 at the same concentration used on day 0. Cells were cultured for another 2 days after which cells were harvested and stored frozen until the Comet assays were performed. These experiments were performed (once in duplicate) for lymphocytes from six different individuals. Cultured cells from wells of the same Se concentration were pooled together into a TV-10 tube on day 9. Cell count was measured.

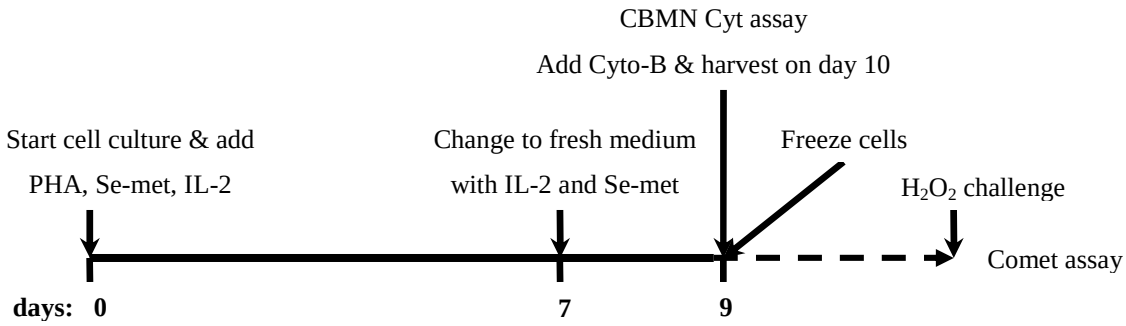


Figure 4-1 Schematic diagram of protocol of long-term culture and Comet assay for testing cytotoxic and genotoxic effects of Se-met concentration.

4.3.6.2 Freezing cultured cells

Cells were resuspended in ice-cold FBS in a 1 ml cryovial with a concentration range of 0.4 – 5 x 10^6 cells/ml, final volumes were either 450 µl or 225 µl depending on the

number of cells available in each culture. Then 50 µl or 25 µl of sterile DMSO was added and mixed well quickly. The cryovial was placed on ice for five minutes, then transferred to a polystyrene foam rack inside a thick-walled polystyrene foam box filled with cotton wool. This was immediately placed in a -80^{o}C freezer for 24 hours before transferring them into liquid nitrogen the next day for long-term storage.

4.3.7 Comet assay

4.3.7.1 Preparation of cell suspension from cryopreserved cells

Cryovials containing frozen lymphocytes were removed from liquid nitrogen and thawed rapidly in a beaker of Milli-Q water pre-warmed to 37^{o}C. After one washing with normal RPMI-1640 culture medium (supplemented with 10% FBS) by centrifuging at 180 g for 10 min., cells were resuspended. To determine cell concentration, cells were counted using a Coulter Counter (Model Z1, Beckman, Australia). Cell concentration was adjusted to 2.0 x 10^5 cells/ml for the Comet assay.

4.3.7.2 Viability count

Cell viability was determined using the Trypan Blue technique. Viable cells do not take up Trypan Blue (Sigma), whereas non-viable cells do. This can be observed under 40x objective of a bright field microscope (Leica 20EB). Initially, 20 µl of cell suspension was added to 20 µl Trypan Blue solution (0.4% w/v, sterile) in an Eppendorf tube. The mixture was then incubated while remaining at room temperature for two minutes then transferred to a haemocytometer. A minimum of 200 cells in one chamber were counted. Non-viable cells stained blue and viable cells remain unstained. Cell viability (%) was calculated as follows:

$$\frac{\text{Total number of viable cells x 100}}{\text{Total number of all counted cells}}$$

4.3.7.3 H_2O_2 treatment

Lymphocytes were challenged with or without 7.5 µM H_2O_2 solution (Sigma, USA) prior to the Comet assay. Briefly, a 7.5 µl H_2O_2 working solution (0.25 mM in RPMI-1640) was added when preparing a total volume of 250 µl of 2.0 x 10^5 cells/ml suspensions. To the controls, 7.5 µl of RPMI-1640 was added instead. Cell suspensions treated with H_2O_2 and controls were incubated at 37^{o}C for 30 minutes prior to the Comet assay, which was performed immediately after the incubation

4.3.7.4 Standard Comet assay

Lymphocyte DNA damage by Comet assay was measured using the alkaline method (Tice et al., 2000), which was modified for use with high throughput CometSlide™ HT (Trevigen Inc. Cat# 4252-02K-01). Cell suspension was combined with 1% low melting agarose gel solution in phosphate buffered saline (PBS, NaCl 8.0 g/l, KCl 0.2 g/l, KH_2PO_4, anhydrous 0.2 g/l, Na_2HPO_4, anhydrous 1.15g/l, pH adjusted to 7.5) which was kept in a 37 ^oC water bath, at a ratio of 1:10, then 75µl x 3 of the mixture was transferred to three of the sample spots on CometSlide™ HT. After agarose gel was set at 4^oC for 30 minutes, slides were immersed in 100ml lysis solution (100mmol/l EDTA disodium salt dehydrate, 2.5mol/l NaCl, 1% N-lauroylsarcosine, 10mmol/l Trizma base, pH adjusted to 10.0), in which 1% Triton X-100 and 10% DMSO was added on the day of experiment and chilled at 4^oC until use. The slides were incubated at 4^oC for 1 hour followed by two washes in MilliQ water for five minutes each. To produce single stranded DNA and to express alkali labile sites (ALS) as single strand breaks, slides were incubated in ice-cold alkaline electrophoresis buffer (1 mmol/l EDTA, 300 mmol/l NaOH, pH adjusted to 13.0) for 25 minutes. Electrophoresis was conducted at 25 V, 450 mA for 20 minutes in the same alkaline buffer in a horizontal Comet assay electrophoresis tank with lid covered (Thistle Scientific; Power supply, Consort E 835). After electrophoresis, slides were washed three times in neutralization buffer (0.4 M Tris-HCl, pH adjusted to 7.5) for 5 minutes to neutralize the alkali in the gels. Slides were then immersed in 70% ethanol for 5 minutes, then air-dried at room temperature overnight. All procedures were performed under a dim light environment but still allowing operations to be performed to avoid light-induced DNA damage. After proper drying, slides were stained in propidium iodide solution (50 µg/ml in PBS) for 10 minutes before scoring and the staining procedure was repeated whenever the dye fluorescence faded. All chemicals were purchased from Sigma, Australia.

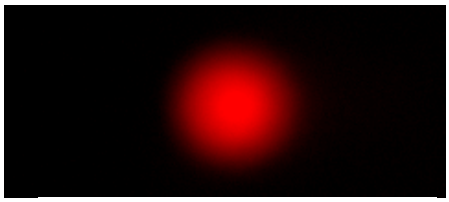
4.3.7.5 Glycosylase treatment

Formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III) were kind gifts of Prof. Andrew Collins' laboratory (Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway) and shipped to Human Nutrition, Adelaide on dry ice. On arrival, enzymes were diluted 100x in enzyme reaction buffer (ERB, 40 mM HEPES (Sigma Cat# H4034), 0.1 M KCL (Sigma Cat# P5405), 0.5 mM EDTA (Sigma Cat# E5134), 0.2 mg/ml Bovine Serum Albumin (Sigma Cat# A7906), pH was adjusted to 8.0 with KOH (Sigma Cat# P1767) and divided into 10 µl aliquots with (Endo III) or without (Fpg) 10% glycerol (Sigma Cat# G5516) and stored at -80^oC.

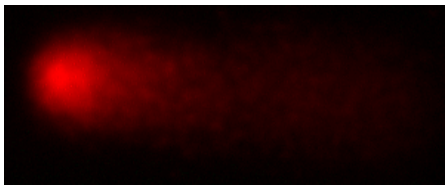
After lysis, slides were washed with enzyme reaction buffer (3 x 5 min). 100x stock enzyme solution was further diluted 4 times with enzyme reaction buffer (i.e. 1:400 extract dilution) before adding to CometSlide with sample loaded (4 µl per sample area). Slides were then incubated at 37^{o}C in a moisturised chamber for 30 minutes and placed at 4^{o}C for a further 5 minutes before immersing in alkaline electrophoresis buffer. The dilution factor used in this study was sufficient to saturate the number of breaks without inducing non-specific cleavage (A. Collins personal communication; (Pitozzi et al., 2006)).

4.3.7.6 Measurement of DNA damage and scoring calibration

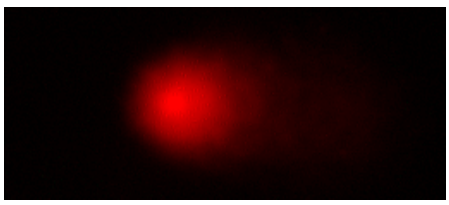
The CometSlide was scored visually using a 20x objective on a fluorescence microscope (Nikon Eclipse E600 with tripleband filter; excitation wavelength 530 nm and emission wavelength 615 nm) after staining in propidium iodide solution (Sigma Cat# P4710, Australia) for 10 minutes in a dark room with dim light. Quantification was achieved by scoring 100 randomly selected comets per sample area (duplicate sample areas), assigning them to one of five DNA damage categories (Figure 4-2), and converting the results to arbitrary units ranging from 0 to 400. Calculation of total arbitrary DNA damage units was based on the formula: DNA damage =Σήί x ί/4, where ήί is the number of cells in the damage degree category ί (0-4) (Collins and Harrington, 2002; Tice et al., 2000).



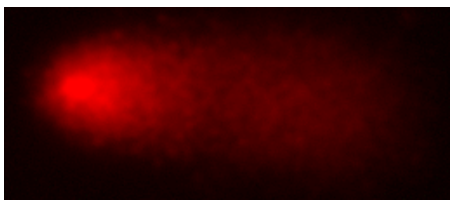
Category 0 DNA damage



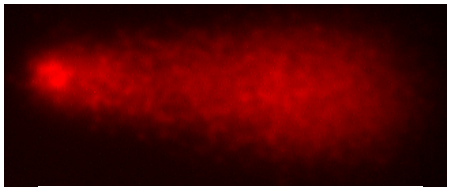
Category 2 DNA damage



Category 1 DNA damage



Category 3 DNA damage



Category 4 DNA damage

Figure 4-2 Images of Comets (from lymphocytes), stained with propidium iodide. These images show typical examples of Comets in categories of 0-4 of DNA damage as used for visual scoring

Scoring was conducted by a single scorer and calibrated against computerized scoring results (CASP 1.2.2 software) using samples exposed to increasing doses of γ radiation. The percentage of DNA in the Comet head and Comet tail (category 0 Comets have 0-5% DNA in tail, category 1 Comets have 5-25% DNA in tail, category 2 Comets have 26-50% DNA in tail, category 3 Comets have 51-75% DNA in tail and category 4 Comets have greater than 75% DNA in tail) was used to construct the γ-ray radiation dose response curve. The Comet score showed a clear linear dose response of total DNA damage to 0, 0.5, 1 and 2 Gy γ-irradiation (Table 4-3). Low dose irradiation mainly induced category 1 DNA damage as shown in Figure 4-3 below.

Table 4-3 Total DNA damage scored as category 0-4 in two Comet assays on cells exposed to a series of γ-ray doses. Between-assay variations was indicated as coefficient of variation (CV%).

γ-irradiation dose (Gy)	Category 0-4 DNA damage(AU)		Average (AU)	CV%
	Assay 1	Assay 2		
0	54	47	50.5	9.8
0.5	79	87	83	6.8
1	94.5	91.5	93	2.3
2	159.5	128.5	144	15.2

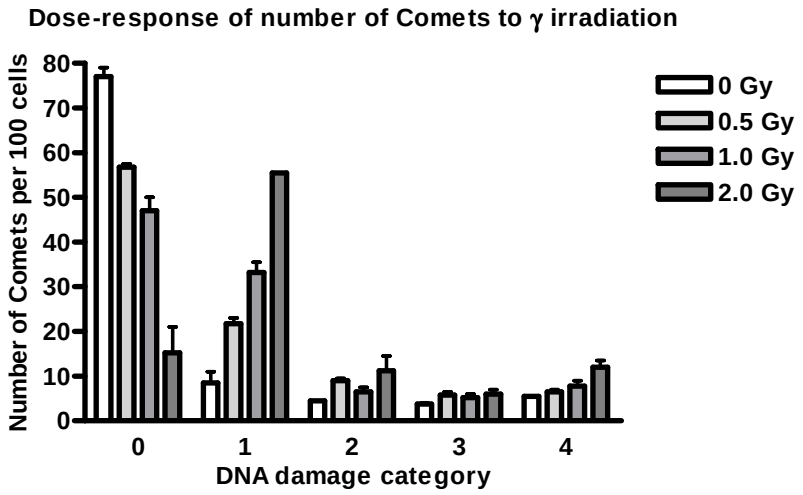


Figure 4-3 Distribution of number of Comets scored (mean of two assays) as category 0-4 DNA damage in response to 0, 0.5, 1.0 and 2.0 Gy of γ-irradiation. Error bars shown are SEMs (N=2).

4.3.8 Statistical analysis

The DNA damage endpoints measured in this study were tested for Gaussian distribution by using the Kolmogorov-Smirnov test. Tests for comparing means of treatment groups were then selected accordingly, i.e. repeated measures one-way ANOVA followed by Tukey's post hoc tests for data with Gaussian distribution and the

non-parametric Friedman test followed by Dunn's multiple comparison test for data that did not exhibit Gaussian distribution. Trend tests for relationship of response variables with Se-met doses were also performed. A $P<0.05$ was considered statistically significant. All of the data are expressed as mean ± standard error (SE). Tests were performed using Prism 4.0 (GraphPad Inc., San Diego, CA) software.

4.3.9 Intra-individual validation of Comet assay with H_2O_2 challenge

To evaluate the reproducibility of the modified Comet assay, six assays were performed on three different days on the frozen lymphocytes isolated from one individual in response to a series of H_2O_2 doses. The Comet DNA damage results and the between assay variations are given in Table 4-4. Viability of thawed cells in each assay ranged from 97% - 99% as determined by the trypan blue assay. The data showed a linear dose-response against H_2O_2 concentrations for category 0 to category 4 DNA damage (Figure 4-4).

The same H_2O_2 dose response validation was performed on frozen lymphocytes of six individuals in one Comet assay. However, these thawed cells isolated from older volunteers were more sensitive to H_2O_2 treatment, i.e. a high percentage of category 4 DNA damage, even when the same batch of reagents was used. Therefore, lower H_2O_2 concentrations (0, 7.5, 15 and 30 µM) were chosen for the experiment. Results are given in Figure 4-5 and Table 4-5. There was a marked difference in baseline DNA damage among 6 individuals within one assay the coefficient of variation (CV) being 21.6% (show in Table 4-5). In comparison to the between-assay variation of baseline DNA damage in one individual (CV % =35.3 in Table 4-4), the between individual variations was smaller, which suggested that day-to-day experimental variability was greater than the variation due to inter-individual difference. The CV was decreased with treatment at increasing concentrations of H_2O_2 in both inter-individual Comet assay and intra-individual Comet assay (range: 5.8-13.4% and 11.8-27.9%, respectively). CV showed that between-assay variations were greater than between individual variations

Table 4-4 Category 0-4 DNA damage (arbitrary unit) scored in six assays on cryopreserved cells of one individual treated with a series of H_2O_2 concentrations. Between assays variations was indicated as coefficient of variation (CV%).

Comet assay	H_2O_2 concentration (µM)				
	0	30	60	90	120
Assay 1	67.5	104	128.5	141	214.5
Assay 2	72.5	98	114.5	150.5	194.5
Assay 3	115.5	125	143.5	172.5	250.5
Assay 4	79	104	138	188	231.5
Assay 5	159.5	192	221.5	248	272.5
Assay 6	126.5	138.5	175	174	226.5
Mean ± SE	103.4 ± 14.9	126.9 ± 14.5	153.5 ± 15.9	179 ± 15.5	231.7 ± 11.1
Between- assay CV%	35.5	27.9	25.4	21.2	11.8

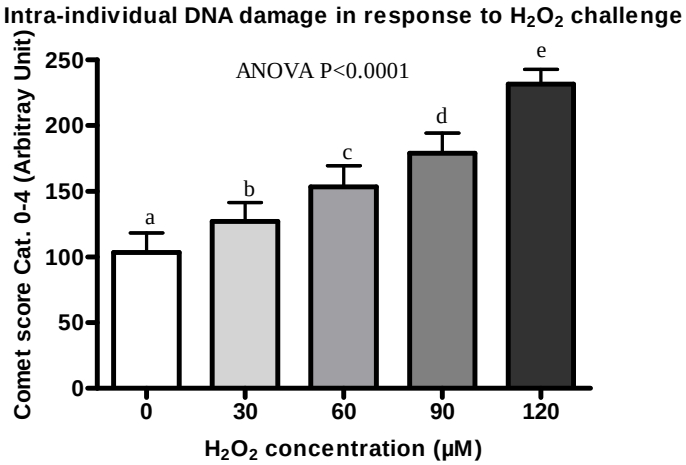


Figure 4-4 H_2O_2 dose response Comet assay: cells from one single individual were treated with a series of H_2O_2 concentrations and repeated in six assays. Columns not sharing the same letter were significantly different from each other.

Table 4-5 Category 0-4 DNA damage (arbitrary unit) scored in one assay on cryopreserved cells of six individuals treated with a series of H_2O_2 concentrations. Between-individual variations are indicated as coefficient of variation (CV%).

Subject	H_2O_2 concentration (µM)			
	0	7.5	15	30
Volunteer 1	35	219	296	327
Volunteer 2	47.5	245	282	324
Volunteer 3	55	291	339	361
Volunteer 4	32	215	283	335
Volunteer 5	44.5	222	336	361
Volunteer 6	35	206	285	315
Mean ± SE	41.5 ± 3.7	233 ± 12.8	305.2 ± 10.4	337.2 ± 8
Between- individual CV%	21.6	13.4	8.4	5.8

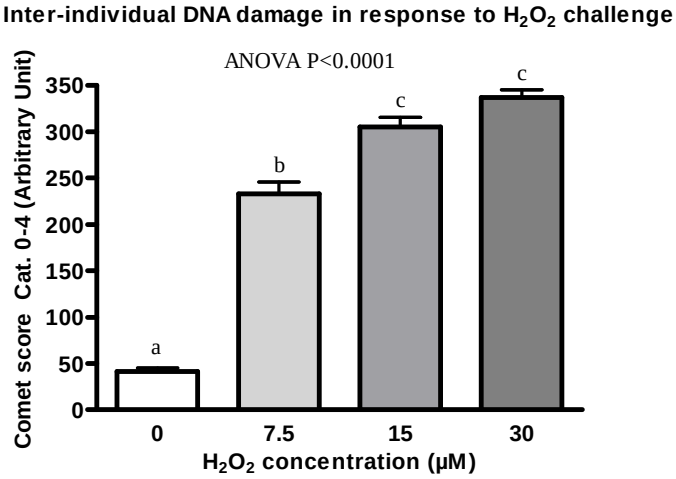


Figure 4-5 H_2O_2 dose response Comet assay: one single assay on cells from six individuals treated with a series of H_2O_2 concentrations. Columns not sharing the same letter were significantly different from each other.

4.4 Results

4.4.1 Plasma Se of volunteers

The characteristics of the volunteers are shown in Table 4-6. The volunteers were healthy non-smoking males aged 28 and 51 years (mean 42.7 yr). The mean plasma Se was 145.2 ± 20.5 µg Se/l, which indicated that on average the volunteers were replete in Se, assuming 120 µg Se/l as the replete cut-off value based on cancer risk minimisation (Combs, 2001b; Combs, 2001a).

Table 4-6 Characteristics of the volunteers

Volunteer profile	
Volunteers	N = 6
Gender	All male
Age, yr, mean (range)	42.7 (28-51)
Plasma Se (µg Se/l), mean (range)	145.2 (93-240)
Multi-vitamin supplement (Se 25 µg/day)	N = 3
Se supplement (200 µg/day)	N = 1
No Se supplement	N = 2
Brazil nut consumption	N = 0

4.4.2 Baseline cell growth and viability of cells

Cell concentrations on day 9 were significantly lower in cultures with the highest Se concentration (3850 µg/l) ($P<0.0001$) (Table 4-7). However, 93 – 99% of the cells were viable in all cultures according to the trypan blue assay. Viability determined by the trypan blue cell count on thawed cells (95 – 98%) on the day of conducting Comet assay was also high for all cultures. The CBMN Cyt assay showed that the rate of necrosis increased at Se concentrations of 1880 and 3850 µg/l but the apoptosis rate was unaffected.

Table 4-7 Cell concentrations, percentage of necrotic and apoptotic cells on day 9 with lymphocytes cultured at six different Se concentrations.

Se concentration of cultures (µg Se/l)	Cell concentration on day 9 (10^6 cells/ml)	Cell death endpoints measured by CBMN Cytome assay	
		necrotic cells %	apoptotic ells %
3	$0.4 \pm 0.05^{a,b,c}$	14.5 ± 1.1^{a}	0.15 ± 0.1
31	0.72 ± 0.11^{a}	$16.8 \pm 3.2^{a,b}$	0.23 ± 0.13
125	$0.54 \pm 0.07^{a,b}$	10.1 ± 1.5^{a}	0.12 ± 0.12
430	$0.56 \pm 0.08^{a,b}$	10.2 ± 1.3^{a}	0.2 ± 0.1
1880	$0.09 \pm 0.01^{b,c}$	24.5 ± 3.8^{b}	0.18 ± 0.1
3850	0.08 ± 0.01^{c}	35.2 ± 3.0^{c}	0.9 ± 0.34
ANOVA P or Friedman P	**<0.0001**	**<0.0001**	0.10

Data were compared across Se concentrations using one-way ANOVA with post-hoc Tukey test or Friedman non-parametric test followed by a Dunn's multiple comparison test. Results are shown as mean ± SE for cultures from five different subjects. Data not sharing the same subscripts were significantly different from each other.

4.4.3 The effect of glycosylase treatment

The comparison of baseline DNA damage of glycosylase treated lymphocytes was based on data pooled from six Se concentrations and five individuals. As one sample was lost due to an accidental event during the thawing procedure, all of the Comet assay results were analysed based on data from five individuals only.

The glycosylase treatment was effective in converting oxidized bases to single strand breaks in the alkaline Comet assay. Glycosylase-treated cells showed significantly greater DNA damage than non-treated cells (P = 0.0002) (Figure 4-6). Glycosylase treatment also appeared to increase the Comet score of DNA damage in cell exposed to H_2O_2 (Figure 4-7), however, the increment was not significant (P=0.098). This may be due to the fact that DNA damage in cells had reached a maximum level following H_2O_2 exposure, particularly for the cultures of high Se concentrations, i.e. 1880 and 3850 µg/l.

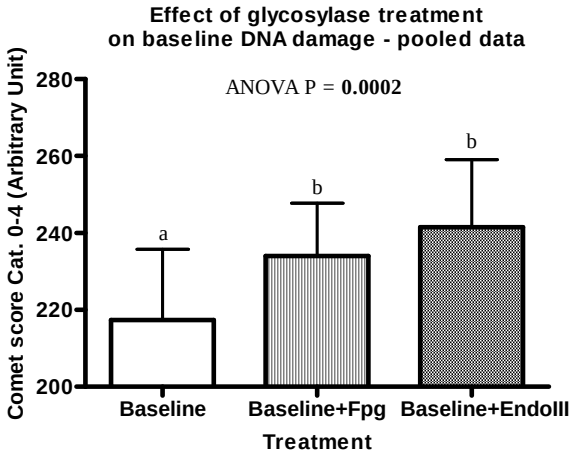


Figure 4-6 Effect of glycosylase treatment (Fpg or Endo III) on baseline DNA damage of cryoperserved human lymphocytes, data pooled from six Se concentrations and five individuals. Data were compared across three treatment groups using one-way ANOVA followed by a post-hoc Tukey test. Columns not sharing the same letter were significantly different from each other.

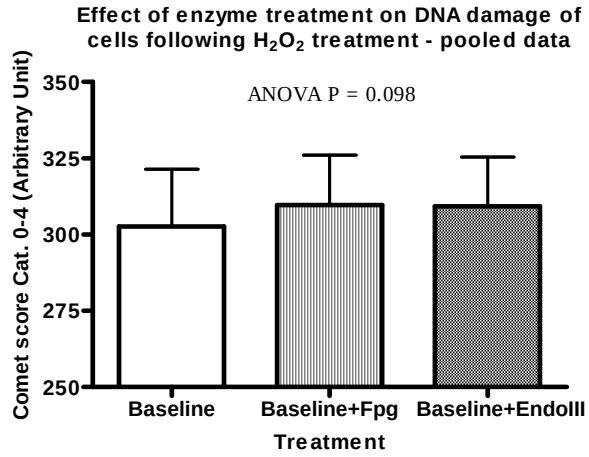


Figure 4-7 Effect of glycosylase treatment (Fpg or Endo III) on DNA damage of cryoperserved human lymphocytes followed by 7.5 µM H_2O_2 treatment, data pooled from six Se concentrations and five individuals. Data were compared across three treatment groups using one-way ANOVA followed by a post-hoc Tukey test.

4.4.4 Baseline DNA damage with or without glycosylase treatment in relation to Se concentration provided as Se-met

The total Comet score of cells, i.e. category 0-4 DNA damage in arbitrary units (Table 4-8), as well as separate score of category 0-3 DNA damage and category 4 DNA damage were reported in the present study (Figure 4-8), so that any apoptotic or necrotic cell with category 4 DNA damage might not confound the results of this study (Fairbairn et al., 1996; Olive et al., 1993).

Baseline category 0-4 DNA damage across cultures of the six Se concentrations was significantly different ($P \leq 0.01$, Table 4-8). This was mainly contributed to by cells cultured at Se 1880 µg Se/l, which had markedly greater category 4 DNA damage than in cells cultured at lower Se concentrations, except 3850 µg Se/l (Figure 4-8). In the latter cultures, some late stage necrotic and apoptotic cells may not be identified and scored due to reduced DNA content levels, which may have biased the DNA damage measured.

Lymphocytes cultured at sub-cytotoxic Se concentrations, i.e. 3-430 µg/l, showed a nonsignificant dose-related trend of lowering in overall baseline DNA damage (category 0-4, $P=0.233$) (Table 4-8), which was mainly due to a dose-related reduction in category 4 cells (Figure 4-8). However, with the glycosylase treatment, a significant trend of increase in category 0-3 DNA damage ($P=0.047$ with Fpg treatment and $P=0.001$ with Endo III treatment, respectively) and a significant trend of lowering in category 4 DNA damage were observed (P trend=0.001 with Endo III treatment) (Figure 4-8).

Table 4-8 DNA damage (arbitrary unit) on day 9 of cryopreserved samples from five different individuals cultured at six Se concentrations, measured by Comet assay (category 0-4) with or without glycosylase Fpg or Endo III treatment.

	Subjects	Selenium (as Se-met) in medium (µg/l)						
		3	31	125	430	1880	3850	P value
Baseline	I	93.5	141	108	143	289	169	**ANOVA 0.0002,** Trend* 0.233
	II	162.0	147	168	186	194	218	
	III	248.0	247	275	194	346	342	
	IV	253.0	200	185	158	316	239	
	V	255.0	258	177	218	340	249	
	Mean	202.3	198.6	182.6	179.8	297	243.4	
	± SE	± 32.3^{a}	± 24.3^{a}	± 26.8^{a}	± 13.3^{a}	± 27.6^{b}	± 28.2^{a,b}	
Baseline + Fpg	I	212	163	215	219	287	181	**Friedman 0.013,** Trend* 0.105
	II	195	166	177	176	207	186	
	III	242	255	291	218	350	325	
	IV	243	200	223	177	288	240	
	V	309	265	200	217	350	241	
	Mean	240.2	209.8	221.2	201.4	296.4	234.6	
	± SE	± 19.5$^{a, b}$	± 21.6^{a}	± 19.1^{a,b}	± 10.2^{a}	± 26.4^{b}	± 26^{a,b}	
Baseline + Endo III	I	218	176	152.	236.	336.	214	**ANOVA 0.0002,** Trend* 0.092
	II	225	153	193	184	245	217	
	III	247	249	283	221	355	334	
	IV	300	205	213	191	300	261	
	V	278	263	179	234	350	232.	
	Mean	253.6	209.2	204	213.2	317.2	251.6	
	± SE	± 15.6^{a,b}	± 20.9^{a}	± 22.1^{a}	± 10.9^{a}	± 20.5^{b}	± 22.2$^{a\ b}$	

Data analysed by using one-way ANOVA with post-hoc Tukey test or Friedman test followed by Dunn's multiple comparison test, and a test for linear trend (N=5). Groups not sharing the same letter are significantly different (P<0.05). * Linear trend test was analysed across Se concentration of 3 -430 µg Se/l only. 100 µg Se as Se-met = 1.25 µM Se-met.

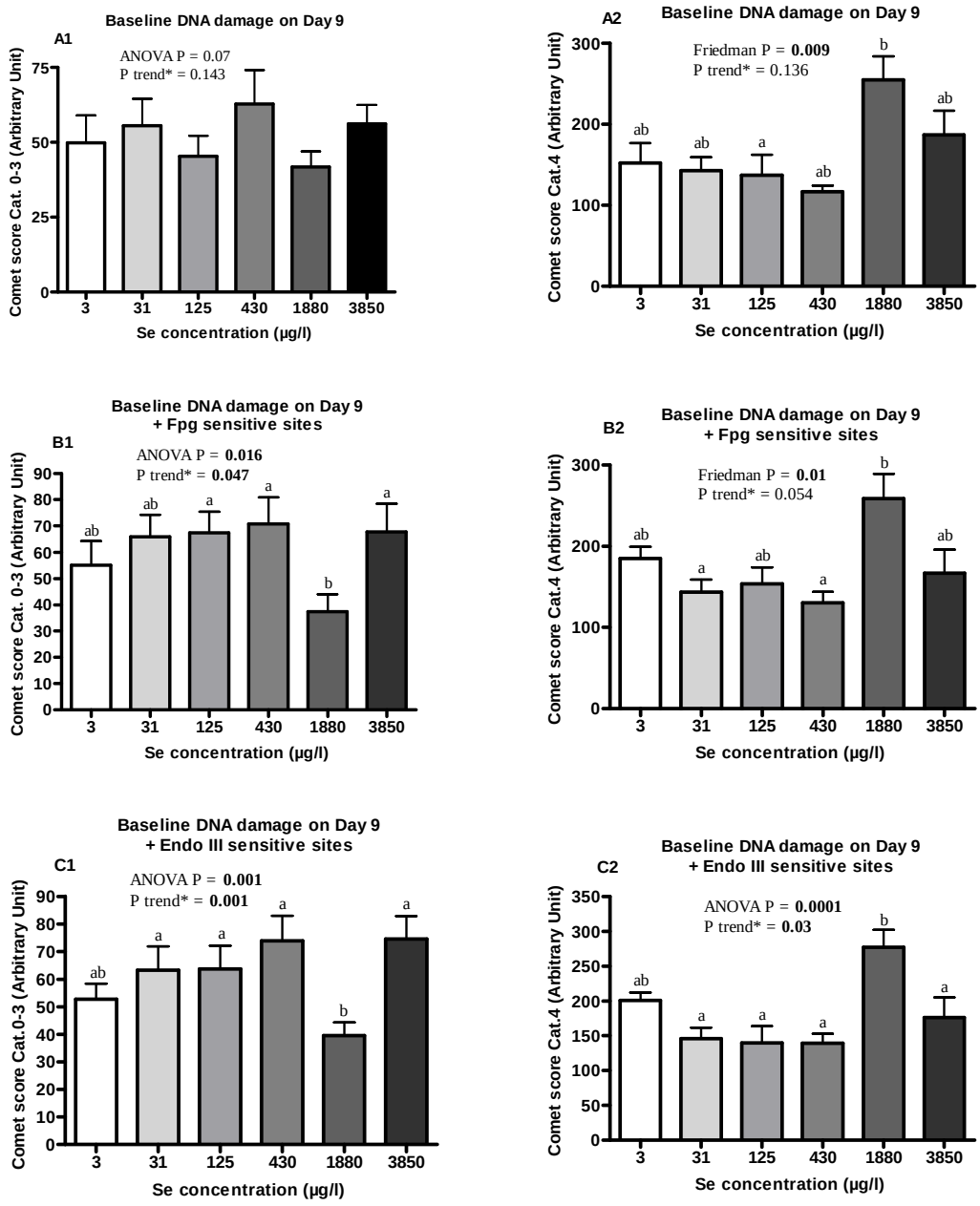


Figure 4-8 Baseline DNA damage on day 9 measured by Comet assay on cryoperserved samples cultured at six Se concentrations: A1, A2 without glycosylase treatment; B1, B2 with Fpg treatment; C1, C2 with Endo III treatment, for catefory 0-3 and category 4 DNA damage, respectively. Data were compared across Se concentrations and between baseline and glycosylase treated using one-way ANOVA with post-hoc Tukey test or Friedman test followed by Dunn's multiple comparison test, and a test for linear trend (N=5). Groups not sharing the same letter are significantly different (P<0.05). * P value for trend was analysed across Se concentration of 3 -430 µg/l only. 100 µg Se as Se-met = 1.25 µM Se-met.

4.4.5 DNA damage with or without glycosylase treatment in H_2O_2 treated cells in relation to Se concentration provided as Se-met

DNA damage in cells challenged with H_2O_2 was also significantly different across six Se concentrations (P≤0.003), but only at the cytotoxic concentration of 1880 µg/l and 3850 µg/l (Table 4-9). With the exception of a significant trend for an increase in H_2O_2-induced category 4 Comet score observed in cells treated with Endo III (P= 0.001, Figure 4-9) as Se concentration increased from 3 to 430 µg Se/l, there was no evidence of a marked impact of Se concentration, in the non-cytotoxic range, on H_2O_2-induced DNA damage Comet score.

Table 4-9 DNA damage (arbitrary unit) on day 9 of five cryopreserved samples cultured at six Se concentrations followed by 7.5 µM H_2O_2 treatment, measured by Comet assay (category 0-4) with or without glycosylase Fpg or Endo III treatment.

	Subjects	Selenium (as Se-met) in medium (µg/l)						
		3	31	125	430	1880	3850	P value
7.5 µM H_2O_2 treated	I	260	234	203	241	362	377	**Friedman 0.003**, Trend* 0.460
	II	219	182	225	245	284	329	
	III	280	331	341	305	381	394	
	IV	329	298	299	258	360	376	
	V	330	311	275	302	377	371	
	Mean ± SE	283.6 ± 21.2^{a,b}	271.2 ± 27.6^{a}	268.6 ± 24.9^{a,b}	270.2 ± 13.9^{a,b}	352.8 ± 17.7^{a,b}	369.4 ± 10.8^{b}	
7.5 µM H_2O_2 treated + Fpg	I	265	261	265	284	356	366	**ANOVA <0.0001**, Trend* 0.567
	II	275	195	248	232	283	343	
	III	296	350	351	321	384	391	
	IV	333	265	280	264	349	377	
	V	308	314	274	311	370	380	
	Mean ± SE	295.4 ± 12.1^{a}	277 ± 26.3^{a}	283.6 ± 17.7^{a}	282.4 ± 16.1^{a}	348.4 ± 17.4^{b}	371.4 ± 8.1^{b}	
7.5 µM H_2O_2 treated + Endo III	I	273	256	280	310	366	379	**ANOVA <0.001**, Trend* 0.895
	II	228	200	239	245	264	333	
	III	318	354	353	311	379	397	
	IV	332	265	293	261	350	377	
	V	308	295	267	300	379	366	
	Mean ± SE	291.8 ± 18.7^{a}	274 ± 25.2^{a}	286.4 ± 18.9^{a}	285.4 ± 13.6^{a}	347.6 ± 21.6^{b}	370.4 ± 10.6^{b}	

Data analysed by using one-way ANOVA with post-hoc Tukey test or Friedman test followed by Dunn's multiple comparison test, and a test for linear trend. Groups not sharing the same letter are significantly different (P<0.05). * Linear trend test was analysed across Se concentration of 3 - 430 µg Se/l only. 100 µg Se as Se-met = 1.25 µM Se-met.

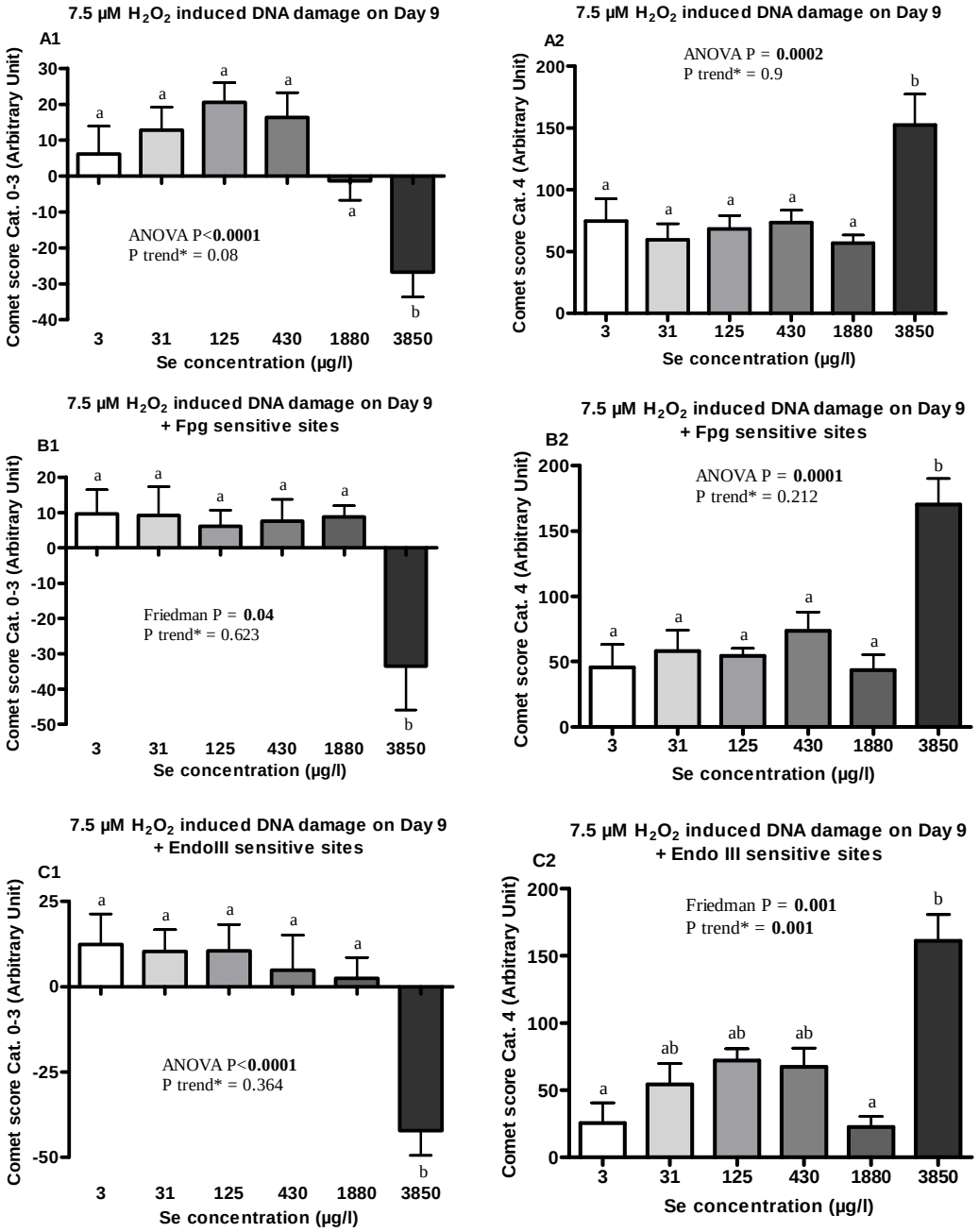


Figure 4-9 DNA damage induced by 7.5 µM H_2O_2 (i.e. measured by subtracting baseline values) on day 9 measured by Comet assay on cryoperserved samples cultured at six Se concentrations: A1, A2 without glycosylase treatment; B1, B2 with Fpg treatment; C1, C2 with Endo III treatment for category 0-3 and category 4, respectively. Data were compared across Se concentrations and between baseline and glycosylase treated using one-way ANOVA with post-hoc Tukey test or Friedman test followed by Dunn's multiple comparison test, and a test for linear trend (N=5). Results represent H_2O_2 induced values measured by subtracting values for non-challenged controls from those of challenged cultures. Groups not sharing the same letter are significantly different (P<0.05). * P value for trend was analysed across Se concentration of 3 -430 µg/l only. 100 µg Se as Se-met = 1.25 µM Se-met.

4.5 Discussion

The findings of this study suggest firstly that Se, as Se-met, is cytotoxic at high concentrations (1880 µg/l or above); secondly, increasing Se concentration within a physiologically relevant range (3-430 µg Se/l) may increase cellular oxidative stress and oxidized DNA damage and thirdly Se-met at all tested subcytotoxic concentrations did not protect cells against H_2O_2 induced oxidative DNA damage.

The present study showed that primary human lymphocyte cultures at 1880 and 3850 µg Se/l had significantly lower cell concentrations on day 9 as well as significantly greater total and category 4 DNA damage as measured by the Comet assay, indicating a cytotoxicity effect of Se-met at high concentrations. This finding is consistent with measurements made using the Cytokinesis-block micronucleus cytome assay, showing necrosis was measured at 1880 and 3850 µg Se/l and was the major form of cell death in Se-met induced cytotoxicity (data shown in Chapter 3 Table 3-6). The mechanisms of Se-met induced cytotoxicity are not fully understood. Some possible mechanisms have been discussed in Chapter 3, including: 1) excessive replacement of methionine by Se-met in proteins may alter protein functions, 2) superoxides generated by Se-met metabolites may increase cellular oxidative stress and oxidative DNA damage and 3) Se-met may affect polyamine metabolism and alter SeAM/SAM ratio and thereby interfere with many biological reactions.

The results of this study showed that Se-met at physiologically relevant concentrations from 31 µg/l (0.4 µM) to 430 µg/l (5.6 µM) had no protective effect against oxidative DNA damage in primary cultured human lymphocytes as measured by the Comet assay. Furthermore, increasing Se concentration in culture medium appeared to increase cellular oxidative stress and oxidative DNA damage when glycosylase-sensitive sites were induced in the Comet assay.

The lack of a protective effect of Se-met against oxidative DNA damage at all concentrations may be explained if maximized GPx activity in lymphocytes was achieved at the lowest Se concentration (i.e. 3 µg/l or 40 nM) used in this study. It has been shown in a few *in vitro* studies that Se compounds could effectively maximize the production and activity of selenoenzymes at quite low concentrations. As a more potent form of Se in terms of increasing selenoprotein activities, selenite at 40-100 nM (3.2 – 8.0 µg Se/l) could induce maximum GPxs and TrxRs activities in several human and

murine cell lines, and thereby, reducing cellular ROS accumulation and DNA damage induced by oxidative challenges (Baliga et al., 2007; Ebert et al., 2006; Leist et al., 1996; Lewin et al., 2002). Although less effective in terms of increasing selenoprotein activities, Se-met as low as 16 µg/l (200 nM) was sufficient to give maximum protection against UV-induced lipid peroxidation and oxidative DNA damage in a few types of human skin cells as measured by the Comet assay (Rafferty et al., 2003; Rafferty et al., 1998) which may be mediated by markedly elevated activity of selenoenzymes. In vitro rat cardiomyocytes culture showed that Se-met at 500 nM (40 µg Se/l) was shown to be the most effective concentration to increase GPx protein and activity (Bordoni et al., 2003). It is also worth mentioning that the cultured lymphocytes were isolated from Se-replete healthy males in this study. The initial Se concentrations within cells may already have been high and the kinetics of cellular Se depletion is unknown. Se in replete cells might be reutilized for the synthesis and function of important selenoenzymes without being affected by a Se deficient environment in the short-term, but after more than 7 days of culture, significant impact of moderate micronutrient deficiency on genome damage has been observed (Crott et al., 2001; Teo and Fenech, 2008). In addition, the observation that nucleoplasmic bridges and nuclear buds were reduced in a dose dependent manner in the 3-430 µg/l concentration range suggests that the DNA lesions measured by the CBMN Cyt biomarkers may be more responsive to subtle benefit of increased Se status than the Comet assay.

Se compounds have been shown to be inducers of ROS and oxidative stress within cells depending on chemical form and concentration (Drake, 2006; Letavayova et al., 2006). It is well documented that selenite is able to generate reactive oxygen species (ROS) and induce oxidative DNA damage and strand break related cell death in a dose-dependent manner starting from as low as 0.25 µM or 20 µg/l (Biswas et al., 2000; Kim et al., 2003; Zhou et al., 2003). Se-met is non-reducible; however, its metabolites, selenide and methylselenol, have strong oxidant potential (Spallholz and Hoffman, 2002; Tarze et al., 2007). Methylselenol was reported to be able to generate a steady state of superoxide by its direct reduction to both dimethyldiselenide and methylseleninic acid in the presence of glutathione (GSH), which may change the cellular levels of oxidative stress (Spallholz et al., 2001). Moreover, Se-met, via its interaction with methioninase, produces superoxide radicals that can lead to further oxidative stress (Spallholz et al., 2004; Zhao et al., 2006). A recent study showed that both Se-met and selenite treatment inhibit growth of null allele yeast strains lacking some nonessential genes in oxidative stress pathways that are involved in the reduction

of ROS (Seitomer et al., 2008). The study suggested that both selenite and Se-met are potentially inducing DNA damage by generating ROS. This is supported in a separate study, which demonstrated 2 µM (160 µg Se/l) of Se-met induced significantly greater DNA damage compared with control and lower concentrations of Se-met as measured by the Comet assay in primary human lymphocyte cultures (Santos and Takahashi, 2008). In the same study, a significantly increased number of chromatid breaks were observed in lymphocytes cultured at a concentration of 0.5 µM (40 µg Se/l) Se-met (Santos and Takahashi, 2008). However, different forms of Se may regulate cellular responses specifically in different cell types. Yeh et al., showed no increase in cellular oxidative stress (cellular GPx/H_2O_2 ratio) or DNA single strand breaks in C6 rat glial cells treated by up to 1 µM (80 µg Se/l) of Se-met (Yeh et al., 2006). Another study reported that methylselenic acid, the direct precursor of methylselenol, at a concentration of up to 5 µM (400 µg Se/l), did not induce DNA damage in mouse mammary hyperplastic epithelial cell lines as measured by the Comet assay (Ip et al., 2000).

H_2O_2 can induce oxidative stress related DNA damage due to its capacity to penetrate the cell and nuclear membrane and initiate the generation of highly reactive species (Deutsch, 1998; Slupphaug et al., 2003). It has been shown that pre-treatment with 1 µM (80 µg Se/l) of Se-met alone did not alter ROS generation in primary cultured rat hippocampal neurons, but significantly protected these cells from iron/hydrogen peroxide induced cell death by increasing cellular GPx activities and decreasing free radical generation (Xiong et al., 2007). However, our study did not show any protective effect of Se-met against H_2O_2-induced DNA breaks and/or oxidized bases at sub-cytotoxic concentrations, i.e. 3 – 430 µg/l, in primary human lymphocytes, which may suggest differences in response between cell types.

In conclusion, Se-met was found in this study to have limited protective effect against oxidative DNA damage in primary cultured human lymphocytes at concentrations up to 430 µg/l. Furthermore, Se-met may increase cellular oxidative stress and can be cytotoxic at doses greater than 430 µg/l. Therefore, a cautious approach to Se supplementation in vitro is required to ensure that optimal genome stability in long term cell cultures is achieved, without undesired effects.

4.6 References

BALIGA, M. S., WANG, H., ZHUO, P., SCHWARTZ, J. L. & DIAMOND, A. M. (2007) Selenium and GPx-1 overexpression protect mammalian cells against UV-induced DNA damage. *Biological Trace Element Research,* 115**,** 227-242.

BISWAS, S., TALUKDER, G. & SHARMA, A. (2000) Chromosome damage induced by selenium salts in human peripheral lymphocytes. *Toxicology In Vitro,* 14**,** 405-408.

BOITEUX, S., GAJEWSKI, E., LAVAL, J. & DIZDAROGLU, M. (1992) Substrate specificity of the Escherichia coli Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionizing radiation or photosensitization. *Biochemistry,* 31**,** 106-110.

BORDONI, A., BIAGI, P. L., ANGELONI, C., LEONCINI, E., MUCCINELLI, I. & HRELIA, S. (2003) Selenium supplementation can protect cultured rat cardiomyocytes from hypoxia/reoxygenation damage. *Journal of Agricultural and Food Chemistry,* 51**,** 1736-1740.

CEMELI, E., BAUMGARTNER, A. & ANDERSON, D. (2008) Antioxidants and the Comet assay. *Mutation Research*.

COLLINS, A. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Molecular Biotechnology,* 26**,** 249-261.

COLLINS, A. & HARRINGTON, V. (2002) Repair of oxidative DNA damage: assessing its contribution to cancer prevention. *Mutagenesis,* 17**,** 489-493.

COLLINS, A. R. (2005) Assays for oxidative stress and antioxidant status: applications to research into the biological effectiveness of polyphenols. *American Journal of Clinical Nutrition,* 81**,** 261S-267S.

COMBS, G., JR. (2005) Current evidence and research needs to support a health claim for selenium and cancer prevention. *Journal of Nutrition,* 135**,** 343-347.

COMBS, G. F., JR. (2001a) Considering the mechanisms of cancer prevention by selenium. *Advances in Experimental Medicine and Biology,* 492**,** 107-117.

COMBS, G. F., JR. (2001b) Impact of selenium and cancer-prevention findings on the nutrition-health paradigm. *Nutrition and Cancer,* 40**,** 6-11.

CROTT, J. W., MASHIYAMA, S. T., AMES, B. N. & FENECH, M. (2001) The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 1089-1096.

DEUTSCH, J. C. (1998) Ascorbic acid oxidation by hydrogen peroxide. *Analytical Biochemistry,* 255**,** 1-7.

DRAKE, E. N. (2006) Cancer chemoprevention: selenium as a prooxidant, not an antioxidant. *Medical Hypotheses,* 67**,** 318-322.

EBERT, R., ULMER, M., ZECK, S., MEISSNER-WEIGL, J., SCHNEIDER, D., STOPPER, H., SCHUPP, N., KASSEM, M. & JAKOB, F. (2006) Selenium supplementation restores the antioxidative capacity and prevents cell damage in bone marrow stromal cells in vitro. *Stem Cells,* 24**,** 1226-1235.

FAIRBAIRN, D. W., WALBURGER, D. K., FAIRBAIRN, J. J. & O'NEILL, K. L. (1996) Key morphologic changes and DNA strand breaks in human lymphoid cells: discriminating apoptosis from necrosis. *Scanning,* 18**,** 407-416.

FENECH, M. (2002) Biomarkers of genetic damage for cancer epidemiology. *Toxicology,* 181-182**,** 411-416.

IP, C., THOMPSON, H., ZHU, Z. & GANTHER, H. (2000) In vitro and in vivo studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. *Cancer Research,* 60**,** 2882-2886.

KECK, A. S. & FINLEY, J. W. (2006) Aqueous extracts of selenium-fertilized broccoli increase selenoprotein activity and inhibit DNA single-strand breaks, but

decrease the activity of quinone reductase in Hepa 1c1c7 cells. *Food and Chemical Toxicology,* 44**,** 695-703.

KIM, T. S., YUN, B. Y. & KIM, I. Y. (2003) Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide. *Biochemical Pharmacology,* 66**,** 2301-2311.

KIMURA, M., UMEGAKI, K., HIGUCHI, M., THOMAS, P. & FENECH, M. (2004) Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *Journal of Nutrition,* 134**,** 48-56.

LEIST, M., RAAB, B., MAURER, S., ROSICK, U. & BRIGELIUS-FLOHE, R. (1996) Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. *Free Radical Biology and Medicine,* 21**,** 297-306.

LETAVAYOVA, L., VLCKOVA, V. & BROZMANOVA, J. (2006) Selenium: from cancer prevention to DNA damage. *Toxicology,* 227**,** 1-14.

LEWIN, M. H., ARTHUR, J. R., RIEMERSMA, R. A., NICOL, F., WALKER, S. W., MILLAR, E. M., HOWIE, A. F. & BECKETT, G. J. (2002) Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell line EAhy926 from damage by lipid hydroperoxides. *Biochimica et Biophysica Acta,* 1593**,** 85-92.

LOFT, S., MOLLER, P., COOKE, M. S., ROZALSKI, R. & OLINSKI, R. (2008) Antioxidant vitamins and cancer risk: is oxidative damage to DNA a relevant biomarker? *European Journal of Nutrition,* 47 Suppl 2**,** 19-28.

MØLLER, P. & LOFT, S. (2002) Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *American Journal of Clinical Nutrition,* 76**,** 303-310.

OLIVE, P. L., FRAZER, G. & BANATH, J. P. (1993) Radiation-induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay. *Radiation Research,* 136**,** 130-136.

PAPP, L. V., LU, J., HOLMGREN, A. & KHANNA, K. K. (2007) From selenium to selenoproteins: synthesis, identity, and their role in human health. *Antioxidants and Redox Signalling,* 9**,** 775-806.

PIPERAKIS, S. M., KONTOGIANNI, K., KARANASTASI, G., IAKOVIDOU-KRITSI, Z. & PIPERAKIS, M. M. (2009) The use of comet assay in measuring DNA damage and repair efficiency in child, adult, and old age populations. *Cell Biology and Toxicology,* 25**,** 65-71.

PITOZZI, V., PALLOTTA, S., BALZI, M., BUCCIOLINI, M., BECCIOLINI, A., DOLARA, P. & GIOVANNELLI, L. (2006) Calibration of the comet assay for the measurement of DNA damage in mammalian cells. *Free Radical Research,* 40**,** 1149-1154.

RAFFERTY, T., GREEN, M., LOWE, J., ARLETT, C., HUNTER, J., BECKETT, G. & MCKENZIE, R. (2003) Effects of selenium compounds on induction of DNA damage by broadband ultraviolet radiation in human keratinocytes. *British Journal of Dermatology,* 148**,** 1001-1009.

RAFFERTY, T. S., MCKENZIE, R. C., HUNTER, J. A., HOWIE, A. F., ARTHUR, J. R., NICOL, F. & BECKETT, G. J. (1998) Differential expression of selenoproteins by human skin cells and protection by selenium from UVB-radiation-induced cell death. *Biochemical Journal,* 332 (Pt 1)**,** 231-236.

REEVES, P. G., GREGOIRE, B. R., GARVIN, D. F., HARELAND, G. A., LINDLAUF, J. E., JOHNSON, L. K. & FINLEY, J. W. (2007) Determination of selenium bioavailability from wheat mill fractions in rats by using the slope-

ratio assay and a modified torula yeast-based diet. *Journal of Agricultural and Food Chemistry,* 55**,** 516-522.

SANTOS, R. A. & TAKAHASHI, C. S. (2008) Anticlastogenic and antigenotoxic effects of selenomethionine on doxorubicin-induced damage in vitro in human lymphocytes. *Food and Chemical Toxicology,* 46**,** 671-677.

SEITOMER, E., BALAR, B., HE, D., COPELAND, P. R. & KINZY, T. G. (2008) Analysis of Saccharomyces cerevisiae null allele strains identifies a larger role for DNA damage versus oxidative stress pathways in growth inhibition by selenium. *Molecular Nutrition and Food Research*.

SLUPPHAUG, G., KAVLI, B. & KROKAN, H. E. (2003) The interacting pathways for prevention and repair of oxidative DNA damage. *Mutation Research,* 531**,** 231-251.

SPALLHOLZ, J., PALACE, V. & REID, T. (2004) Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an in vitro chemiluminescent assay: implications for the nutritional carcinostatic activity of selenoamino acids. *Biochemical Pharmacology,* 67**,** 547-554.

SPALLHOLZ, J. E. & HOFFMAN, D. J. (2002) Selenium toxicity: cause and effects in aquatic birds. *Aquat Toxicol,* 57**,** 27-37.

SPALLHOLZ, J. E., SHRIVER, B. J. & REID, T. W. (2001) Dimethyldiselenide and methylseleninic acid generate superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of L-selenomethionine and L-Se-methylselenocysteine. *Nutrition and Cancer,* 40**,** 34-41.

TARZE, A., DAUPLAIS, M., GRIGORAS, I., LAZARD, M., HA-DUONG, N. T., BARBIER, F., BLANQUET, S. & PLATEAU, P. (2007) Extracellular production of hydrogen selenide accounts for thiol-assisted toxicity of selenite against Saccharomyces cerevisiae. *Journal of Biological Chemistry,* 282**,** 8759-8767.

TCHOU, J., KASAI, H., SHIBUTANI, S., CHUNG, M. H., LAVAL, J., GROLLMAN, A. P. & NISHIMURA, S. (1991) 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proceedings of the National Academy of Sciences of the United States of America,* 88**,** 4690-4694.

TEO, T. & FENECH, M. (2008) The interactive effect of alcohol and folic acid on genome stability in human WIL2-NS cells measured using the cytokinesis-block micronucleus cytome assay. *Mutation Research,* 657**,** 32-38.

TICE, R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. & SASAKI, Y. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* 35**,** 206-221.

XIONG, S., MARKESBERY, W. R., SHAO, C. & LOVELL, M. A. (2007) Seleno-L-methionine protects against beta-amyloid and iron/hydrogen peroxide-mediated neuron death. *Antioxidants and Redox Signalling,* 9**,** 457-467.

YEH, J. Y., OU, B. R., LIANG, Y. C., BURCHFIEL, J., BUTLER, J. A., FORSBERG, N. E. & WHANGER, P. D. (2006) Mechanism for proliferation inhibition by various selenium compounds and selenium-enriched broccoli extract in rat glial cells. *Biometals,* 19**,** 611-621.

ZARCINAS, B., CARTWRIGHT, B. & SPOUNCER, L. (1987) Nitric acid digestion and multi-element analysis of plant material by inductively coupled plasma spectrometry. *Communications in Soil Science and Plant Analysis,* 18**,** 131-146.

ZARCINAS, B., MCLAUGHLIN, M. & SMART, M. (1996) The effect of acid digestion technique on the performance of nebulization systems used in inductively coupled plasma spectrometry. *Communications in Soil Science and Plant Analysis,* 27**,** 1331-1354.

ZHAO, R., DOMANN, F. E. & ZHONG, W. (2006) Apoptosis induced by selenomethionine and methioninase is superoxide mediated and p53 dependent in human prostate cancer cells. *Molecular Cancer Therapeutics,* 5**,** 3275-3284.

ZHOU, N., XIAO, H., LI, T., NUR, E., A, K. & LIU, L. (2003) DNA damage-mediated apoptosis induced by selenium compounds. *Journal of Biological Chemistry,* 278**,** 29532-29537.

5. Genome Health Effect of Wheat Biofortified or Process-fortified with Selenium in Healthy Older Men: a Double-blind Placebo-controlled Trial

5.1 Summary

A supra-nutritional intake of selenium (Se) may reduce the risk of cancer. Genome damage is considered to be a fundamental cause of cancer. The hypothesis that improved Se status by increasing Se intake of wheat process-fortified with selenomethionie (Se-met) (PROFORT) or wheat biofortified with Se (BIOFORT) may reduce DNA damage was tested. This study aimed to compare the bioavailability of Se from PROFORT and BIOFORT high-Se wheat and their impact on antioxidative capacity and genome stability compared to non-fortified normal (CONTROL) wheat in a group of healthy older men. Sixty-two healthy males were randomised into three intervention groups. The CONTROL, BIOFORT and PROFORT groups were provided with biscuits made from low-Se wheat, biofortified high-Se wheat and low-Se wheat fortified with Se-met. Se dosage increased after 8 weeks and again after 16 weeks for a total duration of 24 weeks. Blood samples were collected from participants at baseline and after each 8 weeks of supplementation. Plasma Se concentration, platelet GPx activity and DNA damage (Comet assay) in lymphocytes were investigated. Increased Se intake from BIOFORT wheat increased plasma Se concentration effectively in a dose-response manner from a baseline of 122 µg/l up to 190 µg/l ($P<0.001$). In comparison, Se intake from PROFORT wheat increased plasma Se with a plateau at 140 µg/l, being therefore much less effective than BIOFORT wheat ($P<0.001$). There was no significant change in Se status in the CONTROL group. Improved plasma Se concentrations had no effect on platelet GPx activity or on DNA damage in lymphocytes in all the intervention groups. In conclusion Se from BIOFORT wheat is highly bioavailable. However, the antioxidative capacity and genome stability of this Se-replete group appeared not to be modified by Se supplementation from organic sources.

5.2 Introduction

Selenium is an essential micronutrient for a wide range of biological functions and is represented by at least 25 selenoproteins, such as glutathione peroxidises (GPx), thioredoxin reductases and deiodinases (Kryukov et al., 2003). In the form selenocysteine, Se is incorporated in the active sites of these proteins (Gromer et al., 2005; Hatfield et al., 2006). Therefore, Se is involved in oxidative defence, thyroid hormone metabolism, redox reactions, reproduction and immunity and is important for human health (Rayman, 2000; Rayman, 2002). Recent studies suggest that Se level or intake is implicated in cancer risks, particularly prostate cancer (Meuillet et al., 2004). Some studies suggest that a supra-nutritional intake of Se (200 μg/day) as a supplement, may be required to optimise health (Clark et al., 1996; Clark et al., 1998; Combs and Gray, 1998; van den Brandt et al., 2003). This is three times higher than the intake recommended by the Australian National Health and Medical Research Council (National Health and Medical Research Council, 2006).

Genome instability, resulting from accumulation of DNA damage, has been considered as a fundamental cause of certain degenerative diseases including cancer (Fenech, 2002a; Fenech, 2002b). Micronutrient deficiency or excess can cause genome instability events such as increased rate of micronuclei and elevated rate of aneuploidy (Fenech, 2002c), as demonstrated in the case of iron and folate (Fenech, 2001; Park et al., 2007; Wang et al., 2004). Genome damage is a sensitive and valid biomarker of micronutrient status and is predictive of cancer risk (Fenech, 2003). Improving Se status by increased intake of Se may protect DNA from oxidative damage through improved activity of selenoproteins, such as GPxs. However, excessive intake of Se appeared to increase DNA damage suggesting a U-shaped dose-response (Waters et al., 2003; Waters et al., 2005). Currently, the impact of Se status on genome stability and optimal intake of Se for genome health are unknown.

Selenomethionine (Se-met) is one of the two selenoamino acids naturally occurring in the human diet and is found mostly in plant foods such as wheat (Finley, 2005). Food crops can accumulate Se in their edible parts when Se fertiliser (sodium selenate) is added to the soil or the leaves (Lyons et al., 2004b). Biofortified high-Se wheat is produced by this method in South Australia (Lyons et al., 2005; Lyons et al., 2004b). Wheat is an important dietary source of Se for the Australian population, contributing 30-50% of Se intake (Lyons et al., 2003; Tinggi et al., 1992). Increased intake of

organic selenium from biofortified wheat could improve an individual's selenium status. However, a comparison of the bioavailability and bioefficacy of organic Se from biofortified wheat compared with wheat fortified with pure Se-met during processing is yet to be conducted.

The aims of this study were 1) to compare the bioavailability and bioefficacy of Se from biofortified high-Se (BIOFORT) wheat and from wheat process-fortified post-harvest with L-selenomethionine (PROFORT) and 2) to determine the optimal organic Se intake for prevention of genome damage in a cohort of healthy older men.

5.3 Study design and methods

5.3.1 Recruitment and plasma Se screening of volunteers

All men were recruited from the Adelaide metropolitan and Adelaide Hills area of South Australia through local media advertisement (TV/radio/newspapers) from September to November 2004. Eligible participants were healthy males aged 40-70 years and not supplementing with selenium or above recommended daily intake (RDI) levels of folate and/or vitamin B_{12} and/or vitamin C. The following volunteers were excluded from the study: 1) cancer patients undergoing chemotherapy or radiotherapy; 2) those with sensitivity to study foods, i.e. gluten/wheat intolerance; 3) those unable to comprehend or comply with the study protocol; 4) those not available for all sampling phases of the study. 179 healthy males were screened for Se concentration in plasma and the 81 with lowest plasma Se were recruited to undertake the full trial (February to August, 2005) after giving informed consent. The study was approved by the Human Research Ethics Committee of CSIRO Human Nutrition and the University of Adelaide ethics committee.

There was an unexpected and significant increase in the mean plasma Se concentration measured at the beginning of the trial (122 µg/l) relative to that measured at the screening stage (92 µg/l), which were 2.5 months apart. Analysis of a sub-sample of plasma found that the fluorimetry readings were consistently 22 µg Se/l lower than the mass spectrometry readings. The fluorimetric results appear more consistent with previous Australian studies (Lyons et al., 2004a), hence it is probable that matrix effects increased the apparent Se concentration as determined by mass spectrometry (Lyons NCEFF Se Study Report, 2006). All of the screening samples were analysed by

fluorimetry and all of the trial samples (including the baseline samples) were analysed by mass spectrometry.

5.3.2 Dietary Se intake

A nutrition survey was carried out to investigate the estimated dietary intake of Se in the study cohort and to determine whether the plasma Se concentration is correlated with the estimated Se intake (details are described in Chapter 6). The 179 volunteers who attended the clinic for plasma Se screening prior to the intervention trial were required to complete the validated CSIRO Food Frequency Questionnaire (Baghurst and Record, 1984). Completed questionnaires were analysed by using the FREQUAN dietary analysis program (Baghurst and Record, 1984). The Anti-Cancer Council of Victoria (ACCV) Food Frequency Questionnaire was also completed by volunteers (ACCV, 2003; Ireland et al., 1994). However, the ACCV questionnaire does not provide an estimate of Se intake. The two questionnaires were used for cross validation of food intake estimates.

5.3.3 Intervention design

5.3.3.1 Selenium wheat biscuits used in the trial

The biofortified wheat was grown on the farm of Martin & Kirsty Flower in the South East of South Australia in 2003 using sodium selenate as a foliar spray at around flowering time. The control wheat was low in selenium. The mineral concentrations (other than Se) of three types of study biscuits derived from these low and high Se wheat samples are showed in Table 5-1. The mineral concentrations in the control wheat samples were not different from those in the PROFORT wheat samples. However, the BIOFORT wheat had significantly higher iron and calcium concentrations, and significantly lower concentrations in manganese, copper, zinc, potassium and sulphur.

Table 5-1 Mineral concentrations in three types of study biscuits

Minerals	CONTROL (n=3)	BIOFORT (n=7)	PROFORT (n=7)	One-way ANOVA P
	Mean ± SD (mg/kg)			
Fe	42.3 ± 1.2	54.7 ± 4.7	41.1 ± 2.3	< **0.001**
Mn	68.7 ± 1.5	61.6 ± 1.9	67.3 ± 3.5	**0.001**
B	1.6 ± 0.1	1.6 ± 0.08	1.5 ± 0.08	0.1
Cu	5.3 ± 0.1	4.0 ± 0.1	5.1 ± 0.2	<**0.001**
Mo	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.02	0.28
Zn	24.7 ± 0.6	19.1 ± 0.4	24.7 ± 1.8	<**0.001**
Ca	283.3 ± 5.8	355.7 ± 9.8	272.9 ± 11.1	<**0.001**
Mg	1340 ± 26.5	1385.7 ± 34.1	1322.9 ± 68.0	0.1
Na	20.3 ± 0.6	32.0 ± 1.2	115.9 ± 5.6	<**0.001**
K	4500.0 ± 0	3957.1 ± 78.7	4414.3 ± 203.5	<**0.001**
P	3100.0 ± 0	3285.7 ± 90.0	3071.4 ± 160.4	**0.01**
S	1560.0 ± 20.0	1445.7 ± 17.2	1558.6 ± 25.4	<**0.001**
Al	1.6 ± 0.7	1.3 ± 0.4	1.4 ± 0.4	0.51

The biofortified wheat and process-fortified wheat were delivered as biscuits (developed by Laucke Flour Mills, Strathalbyn, South Australia). Briefly, whole wheat grain were soaked in water to achieve a 16% moisture target over a 12 to 16 hour period in preparation, then heated to expand the grain and then compressed into “puff wheat” biscuits in a rice cake machine. Biscuits process-fortified with selenomethionine (Se-met) were produced by adding pure Se-met (Eburon Organics, USA) to the soaking water, which was absorbed by the grains during processing.

CONTROL, BIOFORT and PROFORT biscuits used in the trial were prepared to be identical in appearance in three separate batches before the intervention commenced. Se content of these biscuits was analysed by Waite Analytical Services (University of Adelaide) throughout the trial and found to remain constant during the study period. The Se concentration, biscuit weight and Se content per biscuit are shown in Table 5-2. The Se concentrations of the BIOFORT and PROFORT biscuits were respectively 123 and 150 times greater than that of the control biscuits. One biofortified biscuit contained 89.1 µg Se compared to 101.9 µg Se in the process-fortified biscuits. These unintended differences were mainly due to different biscuit weights.

Table 5-2 Se concentration, weight and Se content of trial wheat biscuits

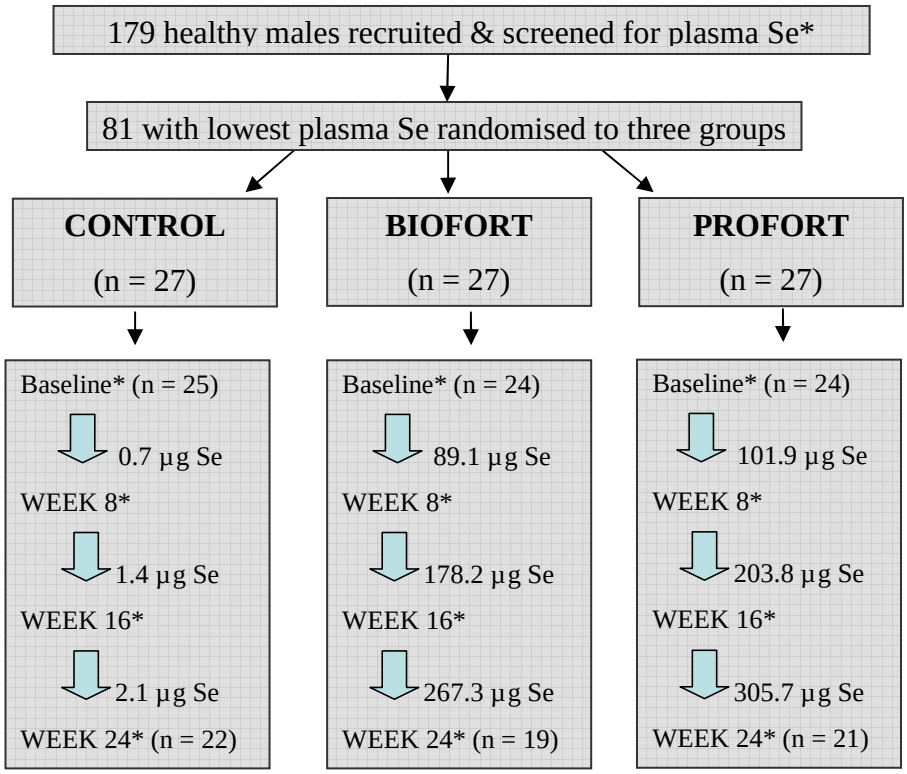
N=7	Se conc. (mg/kg) Mean (range)	Weight (g/biscuit) Mean (range)	Se content (µg/biscuit)
CONTROL wheat	0.07 (0.06-0.08)	10.1 (9.1-10.7)	0.71 (0.64-0.75)
BIOFORT wheat	9.0 (8.7-9.2)	9.9 (9.6-10.5)	89.1 (86.4-94.5)
PROFORT wheat	9.8 (8.7-10.5)	10.4 (9.9-10.8)	101.9 (97.0-105.8)

5.3.3.2 Study protocol

The study was designed as a randomised, double-blind, placebo-controlled trial with a dose-response over a 24-week period from February to August 2005. A total of 73 volunteers recruited were randomised to three groups after eight of the selected 81 withdrew before the trial commenced, as shown in Figure 5-1. The eight withdrawals were due to work commitment or travelling. The dietary groups were designated as CONTROL (low-Se wheat biscuits), BIOFORT (high Se biofortified wheat biscuits) and PROFORT (high Se process-fortified wheat biscuits) according to the type of biscuits they were required to consume. Trial participants consumed one biscuit per day with breakfast during the first eight weeks, then two daily for the next eight weeks, then three biscuits daily for the last eight weeks. During the first eight weeks the BIOFORT and PROFORT groups received approximately an additional 89 or 102 µg/day Se from one biscuit, followed by an additional 178 or 204 µg/day Se in the following eight-week phase from two biscuits and an additional 267 or 306 µg/day Se from three biscuits during the last eight-week phase. The highest Se intake in this trial was higher than the upper limit of intake (National Health and Medical Research Council, 2005) of this mineral (400 µg/day) given the estimated Se intake from food sources of this study cohort was 158 ± 56 (mean ± SD) µg/day (see Chapter 7, Table 7-1). Participants visited the clinic on four occasions to donate a fasting peripheral blood sample before the intervention and at week 8, week 16 and week 24.

Participants were advised to avoid consumption of Brazil nuts or any supplements containing Se, but no additional lifestyle adjustment was needed. To assess compliance participants were required to keep a record of the number of biscuits they had eaten and return any biscuits that were not consumed. Staff directly involved in the trial or in sample analysis and participants were unaware of the selenium level or fortification process of the various biscuits consumed by those participants.

CONTROL, BIOFORT AND PROFORT groups consumed biscuits made from normal wheat, wheat biofortified with selenium and wheat process-fortified with Se-met, respectively. 1, 2 and 3 biscuits were consumed daily between weeks 1-8, weeks 9-16 and weeks 17-24, respectively. The CONTROL, BIOFORT and PROFORT biscuits contained 0.7, 89.1 and 101.9 µg Se each, respectively (Table 5-3).



* Blood samples collected at the beginning of the indicated week.

Figure 5-1 Trial design.

Table 5-3 Estimated daily Se intakes from the intervention biscuits of trial participants at different time-points during the intervention (µg/day) and the percentage of total Se intakes provided by the biscuits (%). Calculation is based on a daily intake of 165 µg Se estimated from FFQ survery study.

Dietary group	Week (biscuits/day)		
	1-8 (1)	9-16 (2)	17-24 (3)
CONTROL	0.7 (0.4%)	1.4 (0.8%)	2.1 (1.2%)
BIOFORT	89.1 (35%)	178.2 (52%)	267.3 (62%)
PROFORT	101.9 (38%)	203.8 (55%)	305.7 (65%)

5.3.4 Blood collection and sample preparation

At each visit, blood was collected in the morning after an overnight fast from each participant by venipuncture into both potassium-EDTA vacutainer (platelets) and lithium-heparin vacutainer (lymphocytes) (Vacuette, Austria) for plasma and cell sample preparation.

5.3.4.1 Isolation and storage of platelets

Nine ml of whole blood was collected into a potassium-EDTA vacutainer. Blood was centrifuged once at 250 g for 10 minutes at room temperature to obtain platelet rich plasma and again at 200 g for five minutes to remove red cells. The platelet rich plasma was washed twice with four ml of cold EDTA/PBS washing buffer (9 mmol/l EDTA, 26.4 mmol/l Na_2HPO_4, 0.14 mmol/l NaCl, 4 ^oC, Sigma, Australia) at 2500 g for 15 minutes at room temperature. Before the second wash, two ml of 0.07 mmol/l ammonium oxalate (Sigma, Australia) was added to lyse the red cells for five minutes. After the final wash, the platelet pellet was resuspended in 1 ml EDTA/PBS cold washing buffer and kept in a cryovial and stored at -80^oC.

5.3.4.2 Isolation and storage of lymphocytes

Two ml of the blood sample collected with lithium-heparin vacutainer tubes was diluted with an equal volume of Hanks balanced salt solution (HBSS, Thermo, US) and mixed. To isolate lymphocytes, 1.4 ml Ficoll-Paque^{TM} (Amersham Pharmacia Biotech), a density gradient medium, was added to a 10 ml TV-10 tube and 4 ml of diluted blood was gently overlaid. The tubes were centrifuged at 400 g for 30 minutes at 22 ^oC. The cells were collected and washed twice first in three volumes and then in two volumes of HBSS followed by centrifugation at 180 g for 10 minutes at room temperature. The cell pellet in the tube was resuspended in one ml of culture medium containing 10% foetal bovine serum (FBS, Trace MultiCel, Australia). A cell count was then conducted using Coulter Counter (Model Z1, Beckman, Australia). One ml of 2 x 10^5 cells/ml suspension was prepared for the Comet assay. Spare cells were spun down and resuspended in 500 µl FBS with 10% DMSO (Sigma, Australia), then stored in liquid nitrogen after freezing at -80^oC for 24 hours.

Lymphocytes isolated from 27 ml of whole blood of one individual using the same method was divided into 300 µl aliquots (3x 10^6 cell/ml) and stored in liquid nitrogen, for use as negative controls in each run of the Comet assay.

5.3.4.3 Plasma Se, folate, Vit B_{12} and homocysteine analysis

Whole blood collected in potassium-EDTA tubes (plasma folate, Vit B_{12} and homocysteine) or Li-heparin tubes (plasma Se) was centrifuged at 2800 x g for 15 minutes at 4^{o}C. 500 µl plasma aliquots were kept at -80^{o}C, sent to Waite Analytical Services (WAS, Adelaide University) for analysis of Se and to the Institute of Medical and Veterinary Science (IMVS), South Australia to measure folate, Vit B_{12} and homocysteine concentrations. Both of these laboratories are nationally certified and accredited laboratories to provide specified analytical measurements for these compounds.

The concentration of plasma Se was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent Technologies 7500c, Japan), following digestion with nitric acid and finishing with hydrochloric acid. There was 1.5% variation from the standard reference (Seronorm Serum Lot JL4409). The plasma folate, vitamin B_{12} and homocysteine analyses were monitored to assess if there were substantial dietary changes during the trial which may have impacted on the DNA damage bio-marker. The plasma folate concentration was measured by using Chemiluminescent Microparticle Folate Binding Protein Assay on the ARCHITECT immunoassay system (Abbott Laboratories, Abott Park, USA) with a total %CV of 2.7-9.5%. The plasma vitamin B_{12} concentration was measure by using a Chemiluminescent Microparticle Intrinsic Factor Assay on the same ARCHITECT system (Abbott Laboratories, USA) with a total %CV of 5.3-9.5%. The plasma homocysteine concentration was measured by using a Fluorescence Polarization Immunoassay (FPIA) on the AxSYM System (Abbott Laboratories, USA) with a total %CV of 2.0-5.1%.

5.3.5 Measure of platelet glutathione peroxidase (GPx) activity

5.3.5.1 Platelet glutathione peroxidase (GPx) assay

Selenoprotein activity was measured by using a GPx assay kit (Sigma, Cat # CGP-1, Australia), which was modified for application as a 96-well microplate spectrophotometric method. Platelets (5.3.4.1) were thawed on ice and disrupted by an ultrasonic sonicator (Soniclean Manufacturer) on high power for 30 minutes in an ice cold water bath. Platelet lysate was then centrifuged (MSE Micro Centaur) at 13,000 rpm for 20 minutes to spin down cell membrane at 4 ^{o}C. The membrane was discarded and the supernatant lysate was kept on ice until analysis. Preparations were kept on ice for about 30 minutes while performing the rest of the procedures. The reagents (120 µl

GPx assay buffer and 10 µl NADPH solution) and platelet lysate were mixed in each well of a microplate and incubated in a microplate reader (SpecraMAX250, Molecular devices) at 25^{o}C for two minutes. Absorbance was read immediately at 340 nm after adding 10 µl of tert-butylhydroperoxide to each well at six time points with 15 seconds initial delay and 30 seconds interval. Reading was performed in triplicate. Blank and controls were included in each reading.

5.3.5.2 Lowry protein assay

The protein content of the platelet lysate was quantified by a direct Lowry protein-dye binding 96-well microplate method. Briefly, 25 µl of platelet lysate and 150 µl of freshly prepared assay reagent (0.1 ml of copper sulphate solution (1% $CuSO_4.5H_2O$) in 10 ml alkaline tartrate solution (Na_2CO_3 20g/l, KNa Tartrate 0.5g/l, NaOH 4g/l) were mixed in each well and stood for 10 min. Then 25 µl of 60% Folin reagent was added. Absorbance was read at 750 nm (SpecraMAX250) 35 minutes later. The quantity of protein was plotted and calculated against a standard curve (0-1000 µg protein/ml) by using SOFTmax PRO software. All chemicals were purchased from Sigma, Australia.

5.3.5.3 Calculation of GPx activity

The GPx activity of platelets was then calculated according to the formulae below and interpreted as oxidation of NADPH (µmol) per minute per mg protein content in platelets.

GPx activity (µmol/min/mg protein) =
(-1) x [ΔA_{sample} - $\Delta A_{control}$] x dilution factor/6.22 x pathlength x mg protein/ml sample

Note: The plate reader gave the ΔA_{sample} and $\Delta A_{control}$ (change in absorbance per minute) from the reaction automatically. Dilution factor = total assay volume/ sample volume = 200 µl/ 60 µl = 3.33. The actual extinction coefficient for NADPH at 340 nm is 6.22 µmol/ml if the pathlength is 1 cm. However, the path length was 0.633 cm when the volume of solution in the well was 200 µl. An adjustment was made to the extinction coefficient according to the path length.

5.3.6 Measurement of DNA damage in lymphocytes

5.3.6.1 Comet assay

Lymphocyte DNA damage by Comet assay was measured using the alkaline method (Tice et al., 2000), which was modified for use with high throughput CometSlide^{TM} HT

(Trevigen Inc. Cat# 4252-02K-01). The assay was conducted on both fresh lymphocytes isolated on the visiting day (5.3.4.2) and on the same cryopreserved lymphocytes. In the latter case, cells of a single participant from four visits were assayed in the same batch to eliminate inter-batch variance. Lymphocytes were thawed on ice and assayed directly after a cell count and viability count.

Cell suspension was combined with 1% low melting agarose gel solution in phosphate buffered saline (PBS, NaCl 8.0 g/l, KCl 0.2 g/l, KH_2PO_4, anhydrous 0.2 g/l, Na_2HPO_4, anhydrous 1.15g/l, pH adjusted to 7.5) which was kept in a 37 ^oC water bath, at a ratio of 1:10, then 75µl x 3 of the mixture was transferred to three of the sample spots on CometSlide HT (Trevigen). After agarose gel was set at 4^oC for 30 minutes, slides were immersed in 100ml lysis solution (100mmol/l EDTA disodium salt dehydrate, 2.5mol/l NaCl, 1% N-lauroylsarcosine, 10mmol/l Trizma base, pH adjusted to 10.0), in which 1% Triton X-100 and 10% DMSO was added on the day of experiment and chilled at 4^oC until use. The slides were incubated at 4^oC for 1 hour followed by two washes in MilliQ water for five minutes each. To express alkali labile sites (ALS) as single strand breaks, slides were incubated in ice-cold alkaline electrophoresis buffer (1 mmol/l EDTA, 300 mmol/l NaOH, pH adjusted to 13.0) for 25 minutes. Electrophoresis was conducted at 25 V, 450 mA for 20 minutes in the same alkaline buffer in a horizontal Comet assay electrophoresis tank with lid covered (Thistle Scientific; Power supply, Consort E 835). After electrophoresis, slides were washed three times in neutralization buffer (0.4 M Tris-HCl, pH adjusted to 7.5) for 5 minutes to neutralize the alkali in the gels. Slides were then immersed in 70% ethanol for 5 minutes, then air-dried at room temperature overnight. All procedures were performed under a dim light environment to avoid light-induced DNA damage. A negative control (5.3.4.2) was included in each assay. After proper drying, slides were stained in propidium iodide solution (50 µg/ml in PBS) for 10 minutes before scoring and the staining procedure was repeated whenever the dye fluorescence faded. All chemicals were purchased from Sigma, Australia.

5.3.6.2 Evaluation of DNA damage

The CometSlide^{TM} was scored visually using 20x objective of a fluorescence microscope (Nikon Eclipse E600 with tripleband filter; excitation wavelength 530 nm and emission wavelength 615 nm for far red) after staining in propidium iodide solution for 10 minutes in a dark room with dim light. Quantification was achieved by scoring 100 randomly selected comets per sample area (duplicate), assigning them to one of five

DNA damage categories (refer Chapter 4, Figure 4-2), and converting the results to arbitrary units ranging from 0 to 400 (Collins, 2002; Tice et al., 2000). Calculation of arbitrary DNA damage units was based on the formula: DNA damage =Σήί x ί/4, where ήί is the number of cells in the damage degree category ί (0-4)

5.3.7 Statistical analysis

5.3.7.1 Sample size

The sample size required to detect a statistically significant difference was predetermined based on the published data on male healthy non-smokers of similar age. For 41 men aged 40-55 yrs, the mean ± 1SD of DNA damage (arbitrary unit, AU) in lymphocytes by Comet assay was 82.3 ± 14.1 (Piperakis et al., 2003). Based on this data, a sample size of 20 subjects per group would have sufficient power to detect a difference of 13 AU of DNA damage in lymphocytes between two groups with 80% power and $P<0.05$, two-tailed, using Prism 4.0 software (GraphPad Inc., San Diego, CA). This level of difference is equivalent to half of the DNA damage induced by 0.8 Gy ^{60}Co (estimated from graph) measured on lymphocytes of 20 healthy males aged 40-55 yr (Tsilimigaki et al., 2003).

5.3.7.2 Statistical analysis

Difference between distributions of smoking and alcohol status of three study groups were determined by Chi-square analysis. One way ANOVA was used to compare the difference of baseline characteristics between treatment groups, such as age, BMI, plasma Se and DNA damage by Comet assay. The significance of changes in time/dose, treatment group and combined time and treatment effects for each parameter (plasma folate, Vit B_{12}, homocysteine, plasma Se, platelet GPx activity and DNA damage) were measured using general linear model repeated measures mixed between-within subjects ANOVA (Pallant, 2005). Baseline variation between groups were adjusted by putting baseline values as covariate in the analysis or using the delta value, i.e. subtract baseline value from all data collected from the same individual, in the analysis. Agreement between the DNA damage data measured by Comet assay on fresh and cryopreserved samples was tested using one sample t-test (two tailed) and Pearson's correlation test. The correlation between plasma Se, folate, Vit B_{12}, homocysteine, platelet GPx activity and DNA damage was determined by Pearson's correlation test based on the baseline data and the pooled data of four visits of each treatment group. Differences with P value <0.05 were considered to be statistically significant. Statistical analyses were performed using the statistical package SPSS for WINDOWS (version 14.0, SPSS Inc, Chicago).

5.4 Results

5.4.1 Characteristics of volunteers

Sixty-two of the original 73 volunteers completed all the intervention with high compliance. Withdrawals during the trial were due to work commitment (n=3), family reason (n=2), travel overseas (n=1), inability to comply (n=2), death (n=1), no reason (n=2). Only results of these 62 subjects were considered in the analysis of the study outcomes.

Characteristics of the 62 participants who completed all stages of the intervention trial are shown in Table 5-4. They were healthy middle-age men with normal BMI. The majority of them were non-smokers (80.9%) and alcohol drinkers (82.6%). Subjects randomized in each of the three study groups were similar in these characteristics with the exception that smoking and alcohol intake varied substantially between groups (Chi square P= 0.06 and P= 0.35, respectively). The mean baseline plasma Se of participants at the start of the intervention was 121.9 ± 12.9 (84-152) µg/l. The mean baseline Se intake of the 62 participants before the intervention was estimated to be 158.4 ± 56.0 µg/day by using CSIRO FFQ. Compliance rate of the study subjects was high (>98%). The estimated intake of Se from the biscuits is given in Table 5-3.

Table 5-4 Characteristics of subjects who completed all stages of the intervention trial (n=62)

	CONTROL N=22	BIOFORT N=19	PROFORT N=21	P value
Age (yrs) Mean ± SD	55.0 ± 7.4	56.1 ± 6.6	55.8 ± 7.1	One way ANOVA 0.86
BMI (m^2/Kg) Mean ± SD	27.0 ± 3.5	26.0 ± 3.3	27.2 ± 3.9	One way ANOVA 0.60
% smokers	18.2	10.5	28.6	Chi-square test 0.06
% drinkers	86.4	94.7	66.7	Chi-square test 0.35
Baseline Se intake (µg/d)*	155.5 ± 39.6	143.3 ± 81.8	174.2 ± 56.0	One way ANOVA 0.22
Baseline plasma Se (µg/l) Mean ± SD	121.0 ± 8.8	122.3 ± 17.1	122.3 ± 12.8	One-way ANOVA 0.94
Compliance rate (%)**	99	99	98	

* The number of participants in control group n=21, BIOFORT group n=18, PROFORT group n=21; ** Reported percentage of biscuits consumed.

5.4.2 Plasma folate, Vit B_{12} and homocysteine level during the whole study

The plasma folate, Vit B_{12} and homocysteine levels of the study subjects measured on the four visits during the study are shown in Table 5-5. The results indicated that the plasma Vit B_{12} levels remained stable during the study. There was time effect for the plasma folate concentrations ($P<0.05$). The effect of time and the effect of time and treatment interaction on plasma homocysteine levels during the trial were significant ($P<0.05$).

Table 5-5 Plasma folate, Vit B_{12} and homocysteine level of study subjects at baseline and after 8, 16 and 24 weeks (WK).

Study group	Baseline	8 WK	16 WK	24 WK	ANOVA P†
	Plasma folate (nmol/l)				
CONTROL N=22	18.7 ± 8.6	18.3 ± 8.0	16.5 ± 7.2	19.2 ± 8.2	**0.04** for time
BIOFORT N=19	17.0 ± 7.9	16.8 ± 6.6	17.4 ± 8.4	17.7 ± 9.4	
PROFORT N=21	19.5 ± 8.3	21.7 ± 8.6	19.7 ± 7.2	20.0 ± 7.9	
ANOVA P†	0.49 for treatment				0.65 for treatment*time
	Plasma Vit B_{12} (pmol/l)				
CONTROL N=22	276.9 ± 110.7	307.6 ± 99.7	294.5 ± 102.1	278.8 ± 107.4	0.92 for time
BIOFORT N=19	284.7 ± 139.5	307.7 ± 157.4	263.8 ± 123.6	247.9 ± 102.0	
PROFORT N=21	299.4 ± 110.6	321.3 ± 126.8	312.2 ± 110.7	290.4 ± 99.9	
ANOVA P†	0.33 for treatment				0.20 for treatment*time
	Plasma homocysteine (µmol/l)				
CONTROL N=22	10.5 ± 8.3	11.2 ± 10.2	10.1 ± 6.0	10.2 ± 7.8	**<0.001** for time
BIOFORT N=19	9.3 ± 2.5	9.2 ± 2.6	9.0 ± 2.3	9.3 ± 2.8	
PROFORT N=21	8.5 ± 1.9	8.6 ± 1.7	8.3 ± 1.3	8.4 ± 1.8	
ANOVA P†	0.24 for treatment				**0.03** for treatment*time

† The statistical analysis was performed using delta value of result of each follow-up time point relative to baseline result and baseline value was included as covariate using mixed between-within subjects ANOVA. Results shown are mean ± SD. P values in bold are statistically significant.

5.4.3 Response of plasma Se to Se supplementation

The plasma Se concentrations at all stages of the trial, by treatment group are presented in Figure 5-2 and Table 5-6. The plasma Se of the placebo group remained unchanged during the whole study period (one-way ANOVA P = 0.19). Subjects in the BIOFORT group had an increasing mean plasma Se from 122.3 µg/l, 144 µg/l, 170.6 µg/l to 193.5 µg/l over 24 weeks (one-way ANOVA P<0.001), corresponding to an 18%, 40% and 59% increment relative to the baseline. The plasma Se concentration also increased in the subjects of the PROFORT group; however, the level reached a peak of 140.5 ± 13.3 µg/l after 16 weeks, which was retained at week 24 (one-way ANOVA P<0.001). The effect of Se supplementation between groups was significantly different (P = 0.007) at weeks 8, 16 and 24. There was also a significant time/dose effect (mixed between-within subjects ANOVA, P = 0.006).

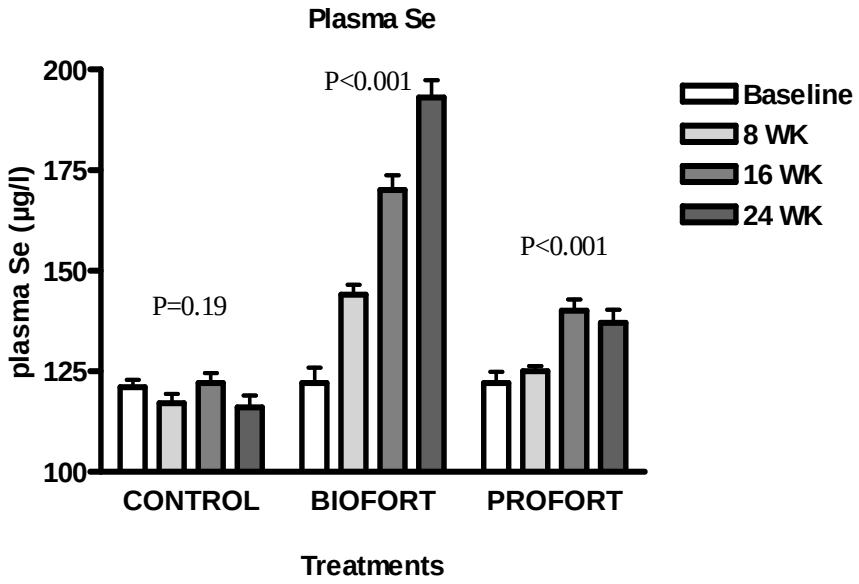


Figure 5-2 Plasma Se concentration during the intervention trial. N = 22, 19, 21 for CONTROL, BIOFORT and PROFORT groups respectively. The error bar shows SEM. One-way ANOVA P values are shown above each of the treatment groups.

Table 5-6 Plasma Se in three treatment groups at baseline and after 8, 16 and 24 weeks (WK).

Study group		Baseline	8 WK	16 WK	24 WK	One-way ANOVA P	Mixed between-within subjects ANOVA P†
		Plasma Se (µg/l)					
CONTROL	Mean	121.1	116.9	122.2	115.7	0.19	
N=22	SD	8.8	10.6	12.2	14.2		
BIOFORT	Mean	122.3	144	170.6	193.5	<0.001	**0.007** for time
N=19	SD	17.1	11.4*	16.5*	18.8*		
PROFORT	Mean	122.3	125.6	140.5	137.9	<0.001	
N=21	SD	12.8	6.1	13.3*	15.2*		
One-way ANOVA P		0.94	0.<0.001	<0.001	<0.001		
Mixed between-within subjects ANOVA P†		**0.006** for treatment					0.13 for treatment*time

† The statistical analysis was performed using delta value of result of each follow-up time point relative to baseline result and baseline value was included as covariate. P values in bold are statistically significant.

5.4.4 Response of selenoenzyme platelet GPx activity to Se supplementation

The results of GPx activity in platelets measured on four visits are as shown in Figure 5-3 and Table 5-7. The activity of GPx in platelets remained unchanged in each of the three groups during the study, regardless of time/dosage or form of Se supplement.

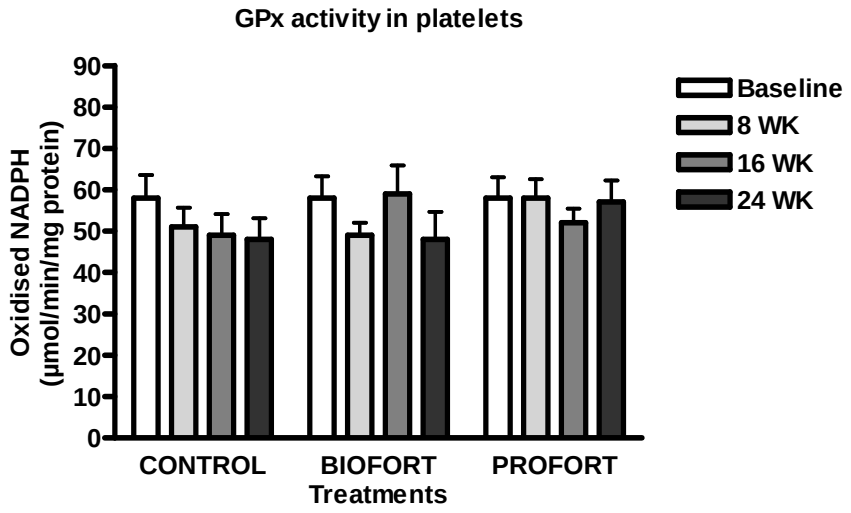


Figure 5-3 Platelet GPx activity during the intervention trial. N = 22, 19, 21 for CONTROL, BIOFORT and PROFORT groups respectively. The error bar shows SEM.

Table 5-7 Platelet GPx activity in three treatment groups at baseline and after 8, 16 and 24 weeks (WK).

Study group		Baseline	8 WK	16 WK	24 WK	One way ANOVA P	Mixed between-within subjects ANOVA P†
		GPx activity in platelets (µmol/min/mg protein)					
CONTROL	Mean	58.0	50.6	49.7	48.3	0.55	0.07 for time
N=22	SD	25.8	22.4	24.5	24.2		
BIOFORT	Mean	57.9	48.9	59.2	48.1	0.39	
N=19	SD	23.1	13.2	30.9	29.2		
PROFORT	Mean	57.9	58.5	52.8	57.2	0.82	
N=21	SD	23.0	21.4	16.8	24.2		
One way ANOVA P		1.0	0.26	0.46	0.44		
Mixed between-within subjects ANOVA P†		0.72 for treatment					0.46 for treatment*time

† The statistical analysis was performed using delta value of result of each follow-up time point relative to baseline result and baseline value was included as covariate.

5.4.5 Response of DNA damage in lymphocytes to Se supplementation

The Comet assay was performed on both the fresh and the cryopreserved lymphocytes. The purpose of repeating the Comet assay on cryopreserved samples was also to assay the samples of the same individual within a single batch-run and minimize the between-batch variation assuming no confounding effects of cryopreservation and frozen storage time. The cryopreserved samples were assayed in 11 batches while the fresh samples were assayed in 24 batches which was dictated by the visiting points.

The DNA damage measured on cryopreserved samples and fresh samples (pooled data of four visits, n = 248) were also compared by using Pearson's correlation test and one sample t-test. The results showed that the two datasets were correlated with an r value of 0.28 (P at 0.01 level, two-tailed), although the DNA damage (category 0-4) measured on cryopreserved samples was significantly less than that measured on fresh samples (mean ± SD were 46.0 ± 20.5 and 66.3 ± 34.1 respectively, $P<0.001$ two-tailed).

Cells with category 0-3 DNA damage and category 4 DNA damage were reported separately (Figure 5-4, Table 5-8, Figure 5-5 and Table 5-9), as cells with category 4 DNA damage are more likely to be apoptotic or necrotic cells in this study which might confound the results for measuring DNA damage in viable cells (Fairbairn et al., 1996; Olive et al., 1993).

Baseline DNA damage of the three treatment groups were not different (one-way ANOVA $P \geq 0.05$). There was a significant time effect ($P < 0.05$) for category 0-3 DNA

damage and category 4 DNA damage in fresh lymphocytes but not in cryopreserved lymphocytes (Table 5-8 and Table 5-9). However, these effects on DNA damage over time did not demonstrate a clear trend of either increase or decline. There was a tendency for results to be highest at week 16 in both fresh and cryopreserved lymphocytes, regardless of treatment (Figure 5-4 and Figure 5-5). Although there was significant treatment effect on category 0-3 DNA damage measured on fresh samples, the effect was not seen when the data was analysed with baseline values as covariate. Overall, the effect of treatment on all category of DNA damage was not significantly different between groups.

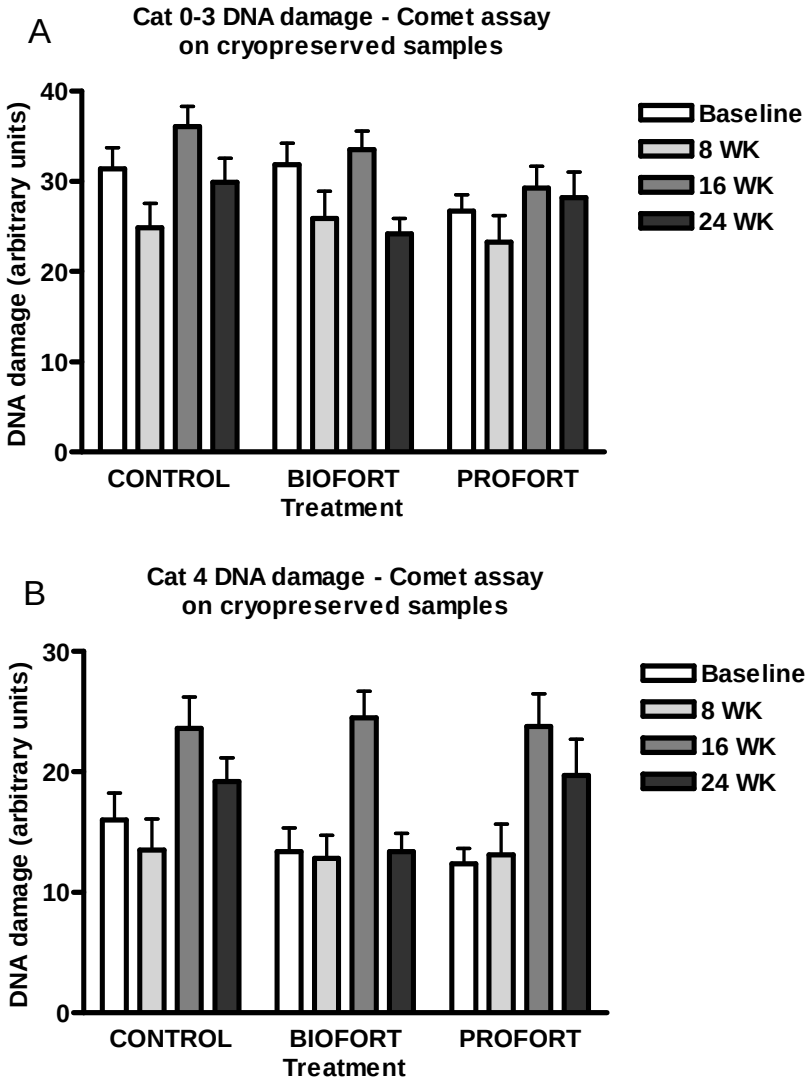


Figure 5-4 DNA damage in cryopreserved lymphocytes measured using the Comet assay. [A] Results for cells showing category 0-3 DNA damage levels. [B] Results for cells showing category 4 DNA damage. N = 22, 19, 21 for CONTROL, BIOFORT and PROFORT groups respectively. The error bar shows SEM.

Table 5-8 Category 0-3 and category 4 DNA damage measured by the Comet assay on cryopreserved samples at baseline and after 8, 16 and 24 weeks (WK).

Study group		Baseline	8 WK	16 WK	24 WK	One way ANOVA P	Mixed between-within subjects ANOVA P†
		Category 0-3 DNA damage (Arbitrary Unit)					
CONTROL	Mean	31.35	24.9	36.1	29.9		
N=22	SD	11.0	12.5	10.4	12.4	0.02	
BIOFORT	Mean	32.0	25.9	33.5	24.2		0.21 for time
N=19	SD	10.2	13.1	9.0	7.5	0.02	
PROFORT	Mean	30.0	23.3	29.3	28.2		
N=21	SD	10.1	13.6	10.9	13.1	0.38	
One way ANOVA P		0.19	0.82	0.10	0.28		
Mixed between-within subjects ANOVA P†		0.19 for treatment					0.47 for treatment*time
		Category 4 DNA damage (Arbitrary Unit)					
CONTROL	Mean	16	13.5	23.5	19.2	0.02	
N=22	SD	10.5	12.1	12.3	9.3		
BIOFORT	Mean	13.7	12.8	24.5	13.4	<0.001	0.06 for time
N=19	SD	8.4	8.4	9.5	6.6		
PROFORT	Mean	12.4	13.1	23.8	19.7	0.004	
N=21	SD	5.7	11.7	12.4	13.8		
One way ANOVA P		0.37	0.98	0.96	0.12		
Mixed between-within subjects ANOVA P†		0.21 for treatment					0.91 for treatment*time

† The statistical analysis was performed using delta value of result of each follow-up time point relative to baseline result and baseline value was included as covariate.

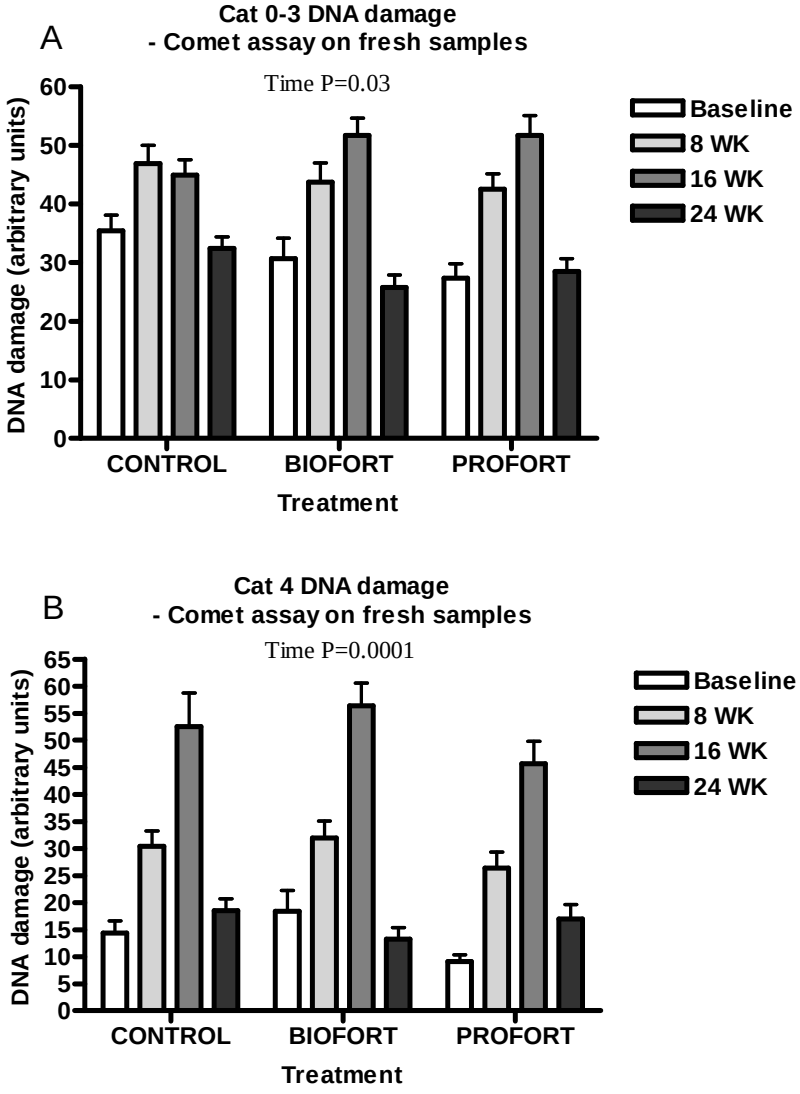


Figure 5-5 DNA damage in lymphocytes measured using the Comet assay. [A] Results for cells showing category 0-3 DNA damage levels. [B] Results for cells showing category 4 DNA damage. N = 22, 19, 21 for CONTROL, BIOFORT and PROFORT groups respectively. The error bar shows SEM.

Table 5-9 Category 0-3 and category 4 DNA damage measured by the Comet assay on fresh samples at baseline and after 8, 16 and 24 weeks (WK).

Study group		Baseline	8 WK	16 WK	24 WK	One way ANOVA P	Mixed between-within subjects ANOVA P†
		Category 0-3 DNA damage (Arbitrary Unit)					
CONTROL	Mean	35.4	46.9	44.9	32.4	<0.001	**0.01** for time
N=22	SD	12.8	14.6	12.5	9.4		
BIOFORT	Mean	30.7	43.7	51.7	25.8	<0.001	
N=19	SD	15.1	14.5	12.8	9.3		
PROFORT	Mean	27.4	42.6	51.7	28.5	<0.001	
N=21	SD	11.0	11.7	15.5	9.9		
One way ANOVA P		0.14	0.57	0.19	0.09		
Mixed between-within subjects ANOVA P†		0.15 for treatment					0.39 for treatment*time
		Category 4 DNA damage (Arbitrary Unit)					
CONTROL	Mean	14.4	30.4	52.5	18.5	<0.001	**<0.001** for time
N=22	SD	10.4	13.2	29.4	10.4		
BIOFORT	Mean	18.4	32	56.4	13.3	<0.001	
N=19	SD	16.9	13.5	18.3	9.1		
PROFORT	Mean	9.1	26.4	45.7	17.0	<0.001	
N=21	SD	5.6	13.5	18.8	12.0		
One way ANOVA P		0.05	0.40	0.33	0.28		
Mixed between-within subjects ANOVA P		0.72 for treatment					0.13 for treatment*time,

† The statistical analysis was performed using delta value of result of each follow-up time point relative to baseline result and baseline value was included as covariate. P values in bold are statistically significant.

5.4.6 Correlation between plasma Se, folate, Vit B_{12}, homocysteine, GPx activity and DNA damage

The correlation matrix values were obtained using the Pearson's correlations test (Table 5-10 and Table 5-11). Baseline data showed that plasma Vit B_{12} concentration was positively correlated to plasma Se and plasma folate concentration (r = 0.51, P at the 0.01 level and r = 0.31, P at the 0.05 level). Baseline plasma Vit B_{12} concentration was also negatively correlated with DNA damage measured by the Comet assay on cryopreserved samples (r= -0.26, P at the 0.05 level and r = -0.34, P at the 0.01 level, for category 0-3 and category 4 DNA damage, respectively). The concentrations of plasma Se (r = -0.26, P at the 0.05 level), Vit B_{12} (r = -0.3, P at the 0.05 level) and folate (r = -0.35, P at the 0.01 level) were negatively correlated to plasma homocysteine concentration. Platelet GPx activity was positively correlated to plasma folate concentration (r = 0.38, P at the 0.01 level) and DNA category 0-3 measured on fresh samples (r = 0.26, P at the 0.05 level).

When the correlation tests were done on pooled data of four visits, the correlation between plasma folate concentration and GPx activity in platelets (r = 0.13, P at the 0.05 level), as well as that between plasma folate concentration and plasma homocysteine concentration (r = -0.36, P at the 0.01 level) were still found. Also, there was a positive correlation between plasma Se and category 4 DNA damage measured on fresh lymphocyte samples (r = 0.13, P at the 0.05 level).

Table 5-10 Pearson correlations coefficient (r) between plasma Se, GPx activity in platelets and DNA damage in lymphocytes by Comet assay on fresh and cryopreserved samples at baseline n = 62

	Plasma folate	Plasma Vit B_{12}	Plasma homocysteine	GPx activity	DNA damage category 0-3, fresh samples	DNA damage category 0-3, cryopreserved samples	DNA damage category 4, fresh samples	DNA damage category 4, cryopreserved samples
Plasma Se	0.19	0.51**	-0.26*	0.01	-0.09	-0.09	0.05	-0.18
Plasma folate		0.31*	-0.35**	0.38**	0.06	-0.12	0.08	-0.23
Plasma Vit B12			-0.30*	0.09	-0.12	-0.26*	-0.02	-0.34**
Plasma homocysteine				0.07	0.13	0.04	0.18	0.17
GPx activity					0.26*	-0.18	0.22	0.04
DNA damage category 0-3, fresh samples						0.17	0.66**	0.42**
DNA damage category 0-3, cryopreserved samples							0.12	0.50**
DNA damage category 4 , fresh samples								0.22

** Correlation was significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Table 5-11 Pearson correlations coefficient (r) between plasma Se, GPx activity in platelets and DNA damage in fresh and cryopreserved lymphocytes by Comet assay for pooled data of four visits of each group n = 248

	Plasma folate	Plasma Vit B_{12}	Plasma homosysteine	GPx activity	DNA damage category 0-3, fresh samples	DNA damage category 0-3, cryopreserved samples	DNA damage category 4, fresh samples	DNA damage category 4, cryopreserved samples
Plasma Se	0.01	0.01	-0.08	0.002	0.02	-0.05	0.13*	0.04
Plasma folate		0.11	-0.36**	0.13*	0.03	-0.11	0.04	-0.08
Plasma Vit B12			-0.28**	0.05	-0.04	-0.09	0.01	-0.04
Plasma homocysteine				0.04	0.03	0.03	-0.04	-0.01
GPx activity					0.05	-0.05	-0.03	0.04
DNA damage category 0-3, fresh samples						0.13*	0.71**	0.27**
DNA damage category 0-3, cryopreserved samples							0.19**	0.63**
DNA damage category 4, fresh samples								0.32**

** Correlation was significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

5.5 Discussion

The present study showed that increasing consumption of biofortified high-Se wheat can increase plasma Se concentration effectively in a time and dose-responsive manner. Plasma Se concentration was raised from 122 µg/l to 144 µg/l, 170 µg/l and 192 µg/l when given 86 µg, 172 µg and 258 µg Se per day from biofortified high-Se wheat for 8 weeks, respectively. This finding is in agreement with previously published reports (Levander et al., 1983; Thomson et al., 1985; van der Torre et al., 1991). In three groups of subjects who had high baseline Se status that was similar to that of our subjects, Meltzer et al. showed that supplementation of 100, 200 or 300 µg Se/day from high-Se bread for 6 weeks increased serum Se levels from 115, 122 130 µg/l to 135, 159 and 183 µg/l, respectively, in three groups of young females in Norway (Meltzer et al., 1992).

Wheat that was process-fortified with Se-met was found to be less bioavailable than biofortified high-Se wheat in this study. Plasma Se concentration reached a maximum of 140 µg/l at week 17 and did not respond to further increase in Se intake of 315 µg per day from process-fortified wheat. This is unexpected as Se-met was reported to be highly bioavailable in terms of improving plasma Se status, even superior to selenized yeast in which the major form of Se is (Burk et al., 2006; Neve et al., 1988). Previous studies had shown no difference in bioavailability of Se from wheat or selenised yeast (Levander et al., 1983; Rayman, 2004). However, studies that compared directly the bioavailability of Se-met and the organic selenium within the wheat grain matrix have not been published. Speciation analysis of the study biscuits after the intervention showed that 88% of the Se in the BIOFORT biscuits was present as selenomethionine while in the PROFORT biscuits only 5% as selenomethionine and 55% of total Se as oxidised selenomethionine (methionine selenoxide) (Table 5-12) (Kirby et al., 2006). Despite high oxidation, the selenomethionine was still somewhat bioavailable. These results suggest that the endogenous Se-met in food is more resistant to oxidation induced by high temperature manufacturing process than the free Se-met added to the biscuits. Although methionine selenoxide can be reduced back to Se-met effectively and rapidly by glutathione in the body (Arteel and Sies, 2001; Sies et al., 1998), its bioavailability appeared to be lower than Se-met as shown in this study. This needs to be confirmed in future intervention studies.

Table 5-12 Total Se, protease-extracted Se and Se species identified in biofortified and process-fortified biscuits (Kirby et al., 2008).

Sample (n=4)	Total Se (µg/g)	Protease extracted [a] (%)	Se-met [b] (%)	Se-O-met [b] (%)
Biofortified wheat biscuits	8.3 ± 0.2	93 ± 3	88 ± 5	-
Process-fortified wheat biscuits	8.5 ± 0.2	91 ± 3	5 ± 1	55 ± 4

[a] Percentage of total Se determined using HNO3. [b] Percentage of total Se extracted using protease.

The lack of effect on the platelet GPx activities by improved plasma Se concentration may be explained by the relatively high baseline plasma Se concentration of the study population (mean 122 µg/l). Such a baseline level is higher than that reported by another survey study in South Australia with a mean plasma level of 103 µg/l (Lyons et al., 2004a). Platelet GPx activities increase rapidly with increasing Se intake (Brown et al., 2000; Neve et al., 1988; van der Torre et al., 1991). However, previous studies have shown that GPx activity in platelets or other blood components is optimised at plasma Se concentration equal to or less than 110-135 µg/l for organic form of Se (Burk et al., 2006; Karunasinghe et al., 2006; Levander et al., 1983; Neve, 1995; Ravn-Haren et al., 2007). When the Se requirement for maximum selenoprotein synthesis is met, increasing Se intake as Se-met is mostly associated with increasing Se concentration in tissues by non-specific incorporation of Se-met in place of methionine, such as albumin in blood (Burk, 2002). There is apparently no regulation of Se-met within the methionine pool (Levander and Burk, 1996). Therefore, very high plasma levels of Se can be achieved when Se-met intake is high and could potentially be toxic for long-term consumption.

Increased intake of Se and plasma Se concentration had no effect on lymphocyte DNA damage as measured by the Comet assay in this study. It has been reported that Se supplementation reduced cancer risks only in participants with baseline plasma Se concentrations below 123 µg/l in the Nutrition Prevention of Cancer Trial (Duffield-Lillico et al., 2003). The plasma Se level of 120 µg/l or greater is considered to be adequate for reducing cancer risks (Combs et al., 2001; Combs, 2005). Such a level may be adequate for reducing oxidative DNA damage as well. This finding is consistent with other studies conducted in Se-replete populations. Increased plasma Se concentration by supplementation of Se-enriched yeast for 9 months did not reduce the urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a biomarker of oxidative DNA damage, in subjects with baseline plasma Se concentration of 127 µg/l (El-Bayoumy et al., 2002). DNA damage in lymphocytes in a group of males at high risk for prostate cancer

has been shown to be inversely associated with serum Se concentration below, but not above 98 µg/l at baseline (Karunasinghe et al., 2004). Results presented here show that the DNA damage in lymphocytes was not correlated to plasma Se concentrations before or after supplementation. Also, a recent study showed that short-term supplementation of organic Se appeared to have no effect on glutathione-mediated detoxification and antioxidation in subjects with baseline serum Se of 115 µg/l (Ravn-Haren et al., 2007). However, DNA stability of subjects with lower baseline Se concentration may still benefit from Se supplementation. A study in *BRCA1* carriers, whose mean baseline Se concentration was 57 µ/l, showed that oral Se supplementation could reduce the chromosome breakage rate in blood lymphocytes (Kowalska et al., 2005). Increased serum Se was found to be protective against oxidative DNA damage as measured by the Comet assay (Dušinská et al., 2002). However, the latter study was performed in a population low in Se intake and may not be applicable to the cohort in our study, which were replete in Se already. Conducting a Se supplementation study in subjects with much lower Se status (e.g. in Serbia) may provide more useful information. There is some evidence that polymorphisms in genes that code for the selenoproteins, such as GPx-1 and selenoprotein P, genes determine the response of selenoprotein markers to Se supplementation and may modify cancer risk (Hu et al., 2005; Hu and Diamond, 2003; Meplan et al., 2007). It would be useful to find out if there is any specific genotype that is more likely to benefit from or be at risk of potential toxic effect from increasing Se intake. It may then be possible, depending on their habitual dietary Se intake, to determine nutritional requirement that is based on an individual's genetic constitution (Fenech, 2007).

Our study showed that increasing plasma Se level up to ~190 µg/l using biofortified wheat does not show any genome toxic effect. The significant fluctuation in lymphocyte DNA damage over time observed in the present study, particularly the increase at 16 weeks, is hard to explain. The observed variation with time in the Comet assay could be due to unknown seasonal changes in exposure to genotoxic environmental factors because DNA damage in the Comet assay has been shown to vary significantly during the year and associated with increased exposure to sunlight, environmental pollutants such as polycyclic aromatic hydrocarbons, changes in exercise level and diet (Dušinská et al., 2002; Møller et al., 1998; Møller et al., 2000; Verschaeve et al., 2007), as indicated by the negative correlation between plasma Vit B_{12} and DNA damage in cryopreserved lymphocytes at baseline; however, Vit B_{12} concentration did not vary

significantly during the 6 month period. The observed variation in plasma folate with time implied changes in diet may have occurred.

In conclusion, BIOFORT high-Se wheat is a good dietary source of Se with high bioavailability in terms of raising plasma Se concentration. However, increased intake of Se or increased plasma Se status in this Se-replete population did not provide any additional health benefits measured using biomarkers of genome stability and antioxidative capacity.

5.6 References

ACCV, A.-C. C. V. (2003) ACCV Dietary Questionnaire. Victoria, Anti-Cancer Council Victoria.

ARTEEL, G. & SIES, H. (2001) The biochemistry of selenium and the glutathione system. *Environmental Toxicology and Pharmacology,* 10**,** 153-158.

BAGHURST, K. I. & RECORD, S. J. (1984) A computerised dietary analysis system for use with diet diaries or food frequency questionnaires. *Community Health Studies,* 8**,** 11-18.

BROWN, K., PICKARD, K., NICOL, F., BECKETT, G., DUTHIE, G. & ARTHUR, J. (2000) Effects of organic and inorganic selenium supplementation on selenoenzyme activity in blood lymphoctyes, granulocytes, platelets and erythrocytes. *Clincal Science,* 98**,** 593-599.

BURK, R. (2002) Selenium, an antioxidant nutrient. *Nutrition in Clinical Care,* 5**,** 75-79.

BURK, R., NORSWORTHY, B., HILL, K., MOTLEY, A. & BYRNE, D. (2006) Effects of chemical form of selenium on plasma biomarkers in a high-dose human supplementation trial. *Cancer Epidemiology, Biomarkers and Prevention,* 15**,** 804-810.

CLARK, L., COMBS, G., JR., TURNBULL, B., SLATE, E., CHALKER, D., CHOW, J., DAVIS, L., GLOVER, R., GRAHAM, G., GROSS, E., KRONGRAD, A., LESHER, J., JR., PARK, H., SANDERS, B., JR., SMITH, C. & TAYLOR, J. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA,* 276**,** 1957-1963.

CLARK, L., DALKIN, B., KRONGRAD, A., COMBS, G., JR., TURNBULL, B., SLATE, E., WITHERINGTON, R., HERLONG, J., JANOSKO, E., CARPENTER, D., BOROSSO, C., FALK, S. & ROUNDER, J. (1998) Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *British Journal of Urology,* 81**,** 730-734.

COLLINS, A. (2002) The Comet Assay - principles, applications, and limitations. IN DIDENKO, V. V. (Ed.) *Methods in molecular biology, in situ detection of DNA damage, methods and protocols.* Totowa, New Jersey, Humana Press.

COMBS, G., JR. (2005) Current evidence and research needs to support a health claim for selenium and cancer prevention. *Journal of Nutrition,* 135**,** 343-347.

COMBS, G., JR. & GRAY, W. (1998) Chemopreventive agents: selenium. *Pharmacology and Therapeutics,* 79**,** 179-192.

COMBS, G. F., JR., CLARK, L. C. & TURNBULL, B. W. (2001) An analysis of cancer prevention by selenium. *Biofactors,* 14**,** 153-159.

DUFFIELD-LILLICO, A. J., DALKIN, B. L., REID, M. E., TURNBULL, B. W., SLATE, E. H., JACOBS, E. T., MARSHALL, J. R. & CLARK, L. C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU International,* 91**,** 608-612.

DUŠINSKÁ, M., VALLOVÁ, B., URSÍNYOVÁ, M., HLADÍKOVÁ, V., SMOLKOVÁ, B., WSÓLOVÁ, L., RAŠLOVÁ, K. & COLLINS, A. (2002) DNA damage and antioxidants: fluctuations through the year in a central European population group. *Food and Chemical Toxicology,* 40**,** 1119-1123.

EL-BAYOUMY, K., RICHIE JR, J., BOYIRI, T., KOMNINOU, D., PROKOPCZK, B., TRUSHIN, N., KLEINMAN, W., COX, J., PITTMAN, B. & COLOSIMO, S. (2002) Influence of selenium-enriched yeast supplementation on biomarkers of oxidative damage and hormone status in helathy adult makes: a clinical pilot study. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 1459-1465.

FAIRBAIRN, D. W., WALBURGER, D. K., FAIRBAIRN, J. J. & O'NEILL, K. L. (1996) Key morphologic changes and DNA strand breaks in human lymphoid cells: discriminating apoptosis from necrosis. *Scanning,* 18**,** 407-416.

FENECH, M. (2001) The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutation Research,* 475**,** 57-67.

FENECH, M. (2002a) Biomarkers of genetic damage for cancer epidemiology. *Toxicology,* 181-182**,** 411-416.

FENECH, M. (2002b) Chromosomal biomarkers of genomic instability relevant to cancer. *Drug Discovery Today,* 7**,** 1128-1137.

FENECH, M. (2002c) Micronutrients and genomic stability: a new paradigm for recommended dietary allowances (RDAs). *Food and Chemical Toxicology,* 40**,** 1113-1117.

FENECH, M. (2003) Nutritional treatment of genome instability: a paradigm shift in disease prevention and in the setting of recommended dietary allowances. *Nutrition Research Reviews,* 16**,** 109-122.

FENECH, M. (2007) Nutrition and genome health. *Forum of Nutrition,* 60**,** 49-65.

FINLEY, J. W. (2005) Selenium accumulation in plant foods. *Nutrition Reviews,* 63**,** 196-202.

GROMER, S., EUBEL, J. K., LEE, B. L. & JACOB, J. (2005) Human selenoproteins at a glance. *Cellular and Molecular Life Sciences,* 62**,** 2414-2437.

HATFIELD, D. L., CARLSON, B. A., XU, X. M., MIX, H. & GLADYSHEV, V. N. (2006) Selenocysteine incorporation machinery and the role of selenoproteins in development and health. *Progress in Nucleic Acid Research and Molecular Biology,* 81**,** 97-142.

HU, Y., BENYA, R. V., CARROLL, R. E. & DIAMOND, A. M. (2005) Allelic loss of the gene for the GPX1 selenium-containing protein is a common event in cancer. *Journal of Nutrition,* 135**,** 3021S-3024S.

HU, Y. J. & DIAMOND, A. M. (2003) Role of glutathione peroxidase 1 in breast cancer: loss of heterozygosity and allelic differences in the response to selenium. *Cancer Research,* 63**,** 3347-3351.

IRELAND, P., JOLLEY, P., GILES, G., O'DEA, K., POWLES, J., RUTISHAUSER, I., WAHLQVIST, M. & WILLIAMS, J. (1994) Development of the Melbourne FFQ: a food frequency questionnaire for use in an Australian prospective study involving an ethnically diverse cohort. *Asia Pacific Journal of Clinical Nutrition,* 3**,** 19-31.

KARUNASINGHE, N., FERGUSON, L. R., TUCKEY, J. & MASTERS, J. (2006) Hemolysate thioredoxin reductase and glutathione peroxidase activities correlate

with serum selenium in a group of New Zealand men at high prostate cancer risk. *Journal of Nutrition,* 136**,** 2232-2235.

KARUNASINGHE, N., RYAN, J., TUCKEY, J., MASTERS, J., JAMIESON, M., CLARKE, L., MARSHALL, J. & FERGUSON, L. (2004) DNA stability and serum selenium levels in a high-risk group for prostate cancer. *Cancer Epidemiology, Biomarkers and Prevention,* 13**,** 391-397.

KIRBY, J., LYONS, G., MARKKAINEN, M. & MCLAUGHLIN, M. (2006) HPLC-(ID)-ICPMS for the determination of selenium species in biofortified grains and biscuits. *INTERACT.* Perth, Australia.

KIRBY, J. K., LYONS, G. H. & KARKKAINEN, M. P. (2008) Selenium speciation and bioavailability in biofortified products using species-unspecific isotope dilution and reverse phase ion pairing-inductively coupled plasma-mass spectrometry. *Journal of Agricultural and Food Chemistry,* 56**,** 1772-1779.

KOWALSKA, E., NAROD, S. A., HUZARSKI, T., ZAJACZEK, S., HUZARSKA, J., GORSKI, B. & LUBINSKI, J. (2005) Increased rates of chromosome breakage in BRCA1 carriers are normalized by oral selenium supplementation. *Cancer Epidemiology, Biomarkers and Prevention,* 14**,** 1302-1306.

KRYUKOV, G. V., CASTELLANO, S., NOVOSELOV, S. V., LOBANOV, A. V., ZEHTAB, O., GUIGO, R. & GLADYSHEV, V. N. (2003) Characterization of mammalian selenoproteomes. *Science,* 300**,** 1439-1443.

LEVANDER, O., ALFTHAN, G., ARVILOMMI, H., GREF, C., HUTTUNEN, J., KATAJA, M., KOIVISTOINEN, P. & PIKKARAINEN, J. (1983) Bioavailability of selenium to Finnish men as assessed by platelet glutathione peroxidase activity and other blood parameters. *American Journal of Clinical Nutrition,* 37**,** 887-897.

LEVANDER, O. & BURK, R. (1996) Selenium. IN ZIEGLER, E. & JR FILER, L. (Eds.) *Present knowledge in nutrition.* 7th ed. Washington, DC, ILSI Press.

LYONS, G., GENC, Y., STANGOULIS, J., LYNDON, T. & GRAHAM, R. (2005) Selenium distribution in wheat grain, and the effect of post-harvest processing on wheat selenium content. *Biological Trace Element Research,* 103**,** 155-168.

LYONS, G., JUDSON, G., STANGOULIS, J., PALMER, L., JONES, J. & GRAHAM, R. (2004a) Trends in selenium status of South Australians. *Medical Journal of Australia,* 180**,** 383-386.

LYONS, G., LEWIS, J., LORIMER, M., HOLLOWAY, R., BRACE, D., STANGOULIS, J. & GRAHAM, R. (2004b) High-selenium wheat: agronomic biofortification strategies to improve human nutrition. *Food, Agriculture & Environment,* 2**,** 171-178.

LYONS, G., STANGOULIS, J. & GRAHAM, R. (2003) Nutriprevention of disease with high-selenium wheat. *Journal of the Australian College of Nutritional and Environmental Medicine,* 22**,** 3-9.

MELTZER, H., NORHEIM, G., LOKEN, E. & HOLM, H. (1992) Supplementation with wheat selenium induces a dose-dependent response in serum and urine of a Se-replete population. *British Journal of Nutrition,* 67**,** 287-294.

MEPLAN, C., CROSLEY, L. K., NICOL, F., BECKETT, G. J., HOWIE, A. F., HILL, K. E., HORGAN, G., MATHERS, J. C., ARTHUR, J. R. & HESKETH, J. E. (2007) Genetic polymorphisms in the human selenoprotein P gene determine the response of selenoprotein markers to selenium supplementation in a gender-specific manner (the SELGEN study). *FASEB Journal,* 21**,** 3063-3074.

MEUILLET, E., STRATTON, S., PRASAD CHERUKURI, D., GOULET, A., KAGEY, J., PORTERFIELD, B. & NELSON, M. (2004) Chemoprevention of prostate cancer with selenium: an update on current clinical trials and preclinical findings. *Journal of Cellular Biochemistry,* 91**,** 443-458.

MØLLER, P., KNUDSEN, L., FRENTZ, G., DYBDAHL, M., WILLIN, H. & NEXØ, B. (1998) Seasonal variation of DNA damage and repair in patients with non-melanoma skin cancer and referents with and without psoriasis. *Mutation Research,* 407**,** 25-34.

MØLLER, P., KNUDSEN, L., LOFT, S. & WALLIN, H. (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiology, Biomarkers and Prevention,* 9**,** 1005-1015.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL, N. (2005) *Nutrition reference values for Australia and New Zealand,* Canberra, Australian government, Department of Health and Aging.

NEVE, J. (1995) Human selenium supplementation as assessed by changes in blood selenium concentration and glutathione peroxidase activity. *Journal of Trace Elements in Medicine and Biology,* 9**,** 65-73.

NEVE, J., VERTONGEN, F. & CAPEL, P. (1988) Selenium supplementation in healthy Belgian adults: response in platelet glutathione peroxidase activity and other blood indices. *American Journal of Clinical Nutrition,* 48**,** 139-143.

OLIVE, P. L., FRAZER, G. & BANATH, J. P. (1993) Radiation-induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay. *Radiation Research,* 136**,** 130-136.

PALLANT, J. (2005) *SPSS survival manual : a step by step guide to data analysis using SPSS for Windows (Version 12),* Crows Nest, N.S.W., Allen & Unwin.

PARK, E., GLEI, M., KNOBEL, Y. & POOL-ZOBEL, B. L. (2007) Blood mononucleocytes are sensitive to the DNA damaging effects of iron overload--in vitro and ex vivo results with human and rat cells. *Mutation Research,* 619**,** 59-67.

PIPERAKIS, S. M., PETRAKOU, E., TSILIMIGAKI, S., SAGNOU, M., MONOGIUDIS, E., HANIOTAKIS, G., KARKASELI, H. & SARIKAKI, E. (2003) Biomonitoring with the comet assay of Greek greenhouse workers exposed to pesticides. *Environmental and Molecular Mutagenesis,* 41**,** 104-110.

RAVN-HAREN, G., BUGEL, S., KRATH, B. N., HOAC, T., STAGSTED, J., JORGENSEN, K., BRESSON, J. R., LARSEN, E. H. & DRAGSTED, L. O. (2007) A short-term intervention trial with selenate, selenium-enriched yeast and selenium-enriched milk: effects on oxidative defence regulation. *British Journal of Nutrition***,** 1-10.

RAYMAN, M. (2000) The importance of selenium to human health. *Lancet,* 356**,** 233-241.

RAYMAN, M. (2002) The argument for increasing selenium intake. *Proceedings of the Nutrition Society,* 61**,** 203-215.

RAYMAN, M. (2004) The use of high-selenium yeast to raise selenium status: how does it measure up? *British Journal of Nutrition,* 92**,** 557-573.

SIES, H., KLOTZ, L. O., SHAROV, V. S., ASSMANN, A. & BRIVIBA, K. (1998) Protection against peroxynitrite by selenoproteins. *Zeitschrift fur Naturforschung. Section C. Biosciences,* 53**,** 228-232.

THOMSON, C., ONG, L. & ROBINSON, M. (1985) Effects of supplementation with high-selenium wheat bread on selenium, glutathione peroxidase and related enzymes in blood components of New Zealand residents. *American Journal of Clinical Nutrition,* 41**,** 1015-1022.

TICE, R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. & SASAKI, Y. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* 35**,** 206-221.

TINGGI, U., REILLY, C. & PATERSON, C. (1992) Determination of selenium in foodstuffs using spectrofluorometry and hudride generation atomic absorption spectrometry. *Journal of Food Composition and Analysis,* 5**,** 269-280.

TSILIMIGAKI, S. I., MESSINI-NIKOLAKI, N., KANARIOU, M. & PIPERAKIS, S. M. (2003) A study on the effects of seasonal solar radiation on exposed populations. *Mutagenesis,* 18**,** 139-143.

VAN DEN BRANDT, P. A., ZEEGERS, M. P., BODE, P. & GOLDBOHM, R. A. (2003) Toenail selenium levels and the subsequent risk of prostate cancer: a prospective cohort study. *Cancer Epidemiology, Biomarkers and Prevention,* 12**,** 866-871.

VAN DER TORRE, H., VAN DOKKUM, W., SCHAAFSMA, G., WEDEL, M. & OCKHUIZEN, T. (1991) Effect of various levels of selenium in wheat and meat on blood Se status indices and on Se balance in Dutch men. *British Journal of Nutrition,* 65**,** 69-80.

VERSCHAEVE, L., KOPPEN, G., GORP, U. V., SCHOETERS, G., JACOBS, G. & ZWIJZEN, C. (2007) Seasonal variations in spontaneous levels of DNA damage; implication in the risk assessment of environmental chemicals. *Journal of Applied Toxicology,* 27**,** 612-620.

WANG, X., THOMAS, P., XUE, J. & FENECH, M. (2004) Folate deficiency induces aneuploidy in human lymphocytes in vitro-evidence using cytokinesis-blocked cells and probes specific for chromosomes 17 and 21. *Mutation Research,* 551**,** 167-180.

WATERS, D. J., SHEN, S., COOLEY, D. M., BOSTWICK, D. G., QIAN, J., COMBS, G. F., Jr., GLICKMAN, L. T., OTEHAM, C., SCHLITTLER, D. & MORRIS, J. S. (2003) Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. *Journal of National Cancer Institute*, 95, 237-241.

WATERS, D. J., SHEN, S., GLICKMAN, L. T., COOLEY, D. M., BOSTWICK, D. G., QIAN, J., COMBS, G. F., Jr. & MORRIS, J. S. (2005) Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model. *Carcinogenesis*, 26, 1256-62.

6. A Survey Study Investigating the Selenium Status, Intake and Dietary Sources in Healthy Older South Australian Men

6.1 Summary

Selenium (Se) is an essential trace element involved in various aspects of human health. This study aimed to determine current dietary Se intake, status and major dietary sources of this element in healthy South Australian men with mean (± SD) age of 55 (± 6.7) years. Food frequency questionnaire (FFQ) studies were conducted to evaluate the dietary Se intake (n=173) and plasma Se concentrations (n=179) in the study population. We found the mean plasma Se concentration of this group of older males was 101.9 µg/l, with the minimum and maximum values of 67 and 156 µg/l, respectively. The estimated mean daily intake of Se was 164.8 µg/d, ranging from 34.6 – 478.1 µg/d. The major dietary sources of Se are bread/cereals, fish/seafood and meat. The findings indicate that the dietary intake of Se in this cohort of older South Australian men was adequate although these results were depending on the Se food composition database of the FFQ used in this study.

6.2 Introduction

Selenium (Se) is one of the essential minerals for human health (Hatfield et al., 2006). As selenocysteine, Se is incorporated into more than 20 selenoproteins in humans, such as glutathione peroxidase (GPx) (Gromer et al., 2005). Severe Se deficiency is rarely observed in Western countries; however, suboptimal Se status has been found to be associated with impaired immune function, reduced fertility and increased cancer risk (Rayman, 2000; Combs, 2005; Etminan et al., 2005). Recent studies suggested a declining trend in blood Se concentration in Australians may be due to soil acidification (Lyons et al., 2005; Lyons et al., 2004). There is limited current information on Se intake and Se status within the Australian populations and the major food sources of Se in South Australian men is unknown. A food frequency questionnaire is one of the most common tools used in food and nutrient intake assessment. The CSIRO Food Frequency Questionnaire (CSIRO FFQ) has been widely used in many dietary survey studies (Baghurst et al., 1988a; Baghurst et al., 1988b); however, the relation between dietary intake of this element estimated using CSIRO FFQ and its blood biomarkers has not been assessed.

The present study aimed to 1) obtain knowledge on the Se intake of the study population by conducting a food frequency questionnaire (FFQ) survey in Australian older men; 2) obtain knowledge on the Se status of the study population by conducting a plasma Se survey in South Australian older men; 3) identify the food groups and nutrients that are associated with Se intake or Se status and 4) evaluate the correlation between biomarkers of Se status and the intake of Se from foods estimated using CSIRO FFQ.

6.3 Study design and methods

6.3.1 Subjects

All men were recruited from the Adelaide metropolitan and the Adelaide Hills areas of South Australia through local media advertisement (TV/radio/newspapers) from September to November 2004. Eligible participants were healthy males aged 40-70 years, not supplementing with selenium or above-RDI levels for folate, vitamin B_{12} and vitamin C. The following volunteers were excluded from the study 1) cancer patients undergoing chemotherapy or radiotherapy; 2) persons who were gluten/wheat intolerant; 3) those unable to comprehend or comply with the study protocol. 179 volunteers were screened for plasma Se concentration following overnight fasting. Volunteers were required to complete two versions of food frequency questionnaires (FFQs) while they attended the clinic. The study was approved by the Human Research Ethics Committee of the CSIRO Division of Human Nutrition and the University of Adelaide ethics committee.

6.3.2 Dietary intake instruments

Two dietary intake instruments in the form of food frequency questionnaires (FFQs) were employed in this study to investigate the estimated dietary intake of nutrients in the study cohort, namely the CSIRO Food Frequency Questionnaire (Baghurst and Record, 1984) and the Anti-Cancer Council of Victoria (ACCV) Dietary Questionnaire (ACCV, 2003). Both questionnaires are self-completed quantitative dietary instruments. Simple instructions on completing the questionnaires were provided. The ACCV FFQ does not provide an estimate of Se intake but is used to identify those nutrients that associated significantly with plasma Se concentration. Only the CSIRO FFQ provides information on intake of Se and food groups.

6.3.2.1 The CSIRO Food Frequency Questionnaire (CSIRO FFQ)

The CSIRO FFQ is a 20-page booklet including a list of over 180 food and beverage items together with qualitative and quantitative questions relating to food preparation practices and dietary habits (Baghurst and Baghurst, 1981). Participants were required to indicate how often a specified serving size of each food and beverage was usually consumed per month, week or day. Using the average daily consumptions of particular foods and the nutrient composition of the food item per unit weight taken from food tables, daily intakes of nutrients of an individual were calculated with the FREQUAN dietary analysis program (Baghurst and Record, 1984). The Australian NUTTAB95 nutrient database and British McCance and Widdowson's food composition tables were consulted as the main sources of information concerning the nutrient composition of foods (Chan et al., 1994; Holland et al., 1993; Holland et al., 1991a; Holland et al., 1992a; Holland et al., 1992b; Holland et al., 1991b; Ministry of Agriculture Fisheries and Food, 1998). Where these data bases did not contain information on the food, or for particular nutrients not covered, such as Se content in Brazil nut, equivalent data were used from the food composition tables of the US Department of Agriculture of US (USDA) (Gebhardt et al., 2007).

This CSIRO FFQ was first developed in the 1980s and has been used extensively with Australian population samples in epidemiological and national survey work (Baghurst et al., 1988a; Baghurst et al., 1988b). This FFQ has been shown to have a high repeatability and consistency with other dietary intake measurement techniques, such as weighed food record and multiple repeat 24-h records, and was demonstrated to have good reliability when correlated with urinary sodium and nitrogen measures (Baghurst and Record, 1983; Lazarus et al., 1995; Rohan and Potter, 1984; Rohan et al., 1987). It is regularly updated to reflect the trends in dietary habits. In the present study, one question regarding Brazil nut consumption was added to capture the Se intake from this important source of Se.

6.3.2.2 The Anti-Cancer Council Victoria Food Frequency Questionnaire (ACCV FFQ)

The four page format ACCV FFQ includes 74 food items with responses on a 10-point scale (1 = Never to 10 = 3 or more times per day) in the frequency section and detailed questions on specific foods over the previous 12 months (Ireland et al., 1994). A series of photographs is included to enable estimation of portion size. Also, 10 cross-check questions are used to adjust for overestimation of fruit and vegetable consumption. The

completed ACCV FFQs are optically scanned and nutrient intakes are computed from FFQ responses using software developed by the ACCV based on the NUTTAB95 nutrient composition data (Ireland et al., 1994). Vitamin E and folate intake are computed using the British McCance and Widdowson's database. However, the analysis does not provide information on the estimated intakes of Se and Vit B_{12}.

The ACCV FFQ was initially developed in the late 1980s to measure dietary intake for use in an ethnically diverse cohort of Australian-born men and women (Ireland et al., 1994). The current ACCV FFQ has been validated against weighed food records and found to be reliable (Hodge et al., 2000). It has been used in a number of large epidemiological studies in the Australian population (Ball et al., 2003; Martin et al., 2007; Woods et al., 2002).

6.3.3 Determination of plasma Se

Eight ml of whole blood collected in a potassium-EDTA tube from each volunteer was centrifuged at 2800 x g for 15 min at 4^{o}C to obtain plasma. 500 µl of plasma was removed and kept at -80^{o}C until sent for Se concentration analysis at the South Australia Research and Development Institute (SARDI). Se was analysed by the fluorimetric method involving the reaction of 2, 3-diaminonaphthalene (DAN) with Se (IV) to form a fluorescent Se-DAN complex, piazselenol (Koh and Benson, 1983). Following the acid digestion and addition of DAN, the fluorescent complex is extracted using the organic solvent cyclohexane. The fluorescence is then measured by fluorometer (Hitachi F2000, Tokoy) (excitation wave length 364 nm and emission wave length 523 nm). Se concentration is calculated against a series of standards according to Koh and Benson (Koh and Benson, 1983). Quality control included the analysis of Seronorm standards and analysis of 21 duplicate samples, which varied by a mean of 1.8% (max. 4%).

6.3.4 Determination of platelet glutathione peroxidase (GPx) activity

6.3.4.1 Platelet glutathione peroxidase (GPx) assay

Selenoprotein activity was measured by using a GPx assay kit (Sigma, Cat # CGP-1, Australia), which was modified for application as a 96-well microplate spectrophotometric method. Platelets (5.3.4.1) were thawed on ice and disrupted by an ultrasonic sonicator (Soniclean Manufacturer) on high power for 30 minutes in an ice cold water bath. Platelet lysate was then centrifuged (MSE Micro Centaur) at 13,000 rpm for 20 minutes to spin down cell membrane at 4 ^{o}C. The membrane was discarded

and the supernatant lysate was kept on ice until analysis. Preparations were kept on ice for about 30 minutes while performing the rest of the procedures. The reagents (120 µl GPx assay buffer and 10 µl NADPH solution) and platelet lysate were mixed in each well of a microplate and incubated in a microplate reader (SpecraMAX250, Molecular devices) at 25^{o}C for two minutes. Absorbance was read immediately at 340 nm after adding 10 µl of tert-butylhydroperoxide to each well at six time points with 15 seconds initial delay and 30 seconds interval. Reading was performed in triplicate. Blank and controls were included in each reading.

6.3.4.2 Lowry protein assay

The protein content of the platelet lysate was quantified by a direct Lowry protein-dye binding 96-well microplate method. Briefly, 25 µl of platelet lysate and 150 µl of freshly prepared assay reagent (0.1 ml of copper sulphate solution (1% $CuSO_4.5H_2O$) in 10 ml alkaline tartrate solution (Na_2CO_3 20g/l, KNa Tartrate 0.5g/l, NaOH 4g/l) were mixed in each well and stood for 10 min. Then 25 µl of 60% Folin reagent was added. Absorbance was read at 750 nm (SpecraMAX250) 35 minutes later. The quantity of protein was plotted and calculated against a standard curve (0-1000 µg protein/ml) by using SOFTmax PRO software. All chemicals were purchased from Sigma, Australia.

6.3.4.3 Calculation of GPx activity

The GPx activity of platelets was then calculated according to the formulae below and interpreted as oxidation of NADPH (µmol) per minute per mg protein content in platelets.

GPx activity (µmol/min/mg protein) =

(-1) x [ΔA_{sample} - $\Delta A_{control}$] x dilution factor/6.22 x pathlength x mg protein/ml sample

Note: The plate reader gave the ΔA_{sample} and $\Delta A_{control}$ (change in absorbance per minute) from the reaction automatically. Dilution factor = total assay volume/ sample volume = 200 µl/ 60 µl = 3.33. The actual extinction coefficient for NADPH at 340 nm is 6.22 µmol/ml if the pathlength is 1 cm. However, the path length was 0.633 cm when the volume of solution in the well was 200 µl. An adjustment was made to the extinction coefficient according to the path length.

6.3.5 Statistical analysis

All of the correlation relationships were determined using a two-tailed Pearson's correlation test. P values less than 0.05 were considered statistically significant. A multiple regression model was also used to identify the food groups that are likely to

explain most of the varieties in plasma Se status. Statistical analyses were performed using the statistical package SPSS for WINDOWS (version 16.0, SPSS Inc, Chicago).

A total of 179 subjects provided a blood sample for plasma Se screening and completed the two dietary intake questionnaires. Six CSIRO FFQs and 14 ACCV FFQs were not completed properly and these were rejected during the analysis process. Therefore, 173 CSIRO FFQs and 165 ACCV FFQs were included in the analysis.

6.4 Results

6.4.1 Se intake measured by CSIRO FFQ and plasma Se status

The plasma Se concentration of the study cohort, healthy males with mean age (± SD) of 55 (± 6.7) years, was 101.9 ± 11.8 µg/l (mean ± SD) (n=179). Using the fluorimetric assay, it appears that only 7% of this group of men had plasma Se concentration greater than 120 µg/l, which is the plasma concentration above which cancer risk may be minimised (Combs et al., 2001). The estimated daily intake of Se was 164.8 ± 68.3 µg/day (mean ± SD) as estimated by the CSIRO FFQ (Figure 6-1).

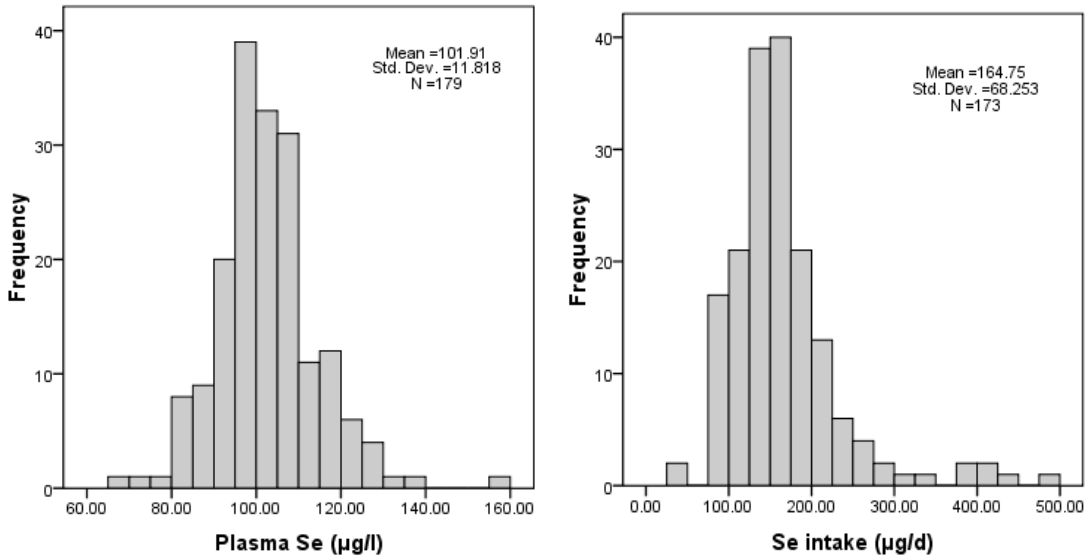


Figure 6-1 Distribution of plasma Se concentration (n=179) and estimated Se intake (n=173) of the study population.

6.4.2 Total Se intake measured by CSIRO FFQ and its correlation with plasma Se status

The energy-adjusted Se intake (µg/kJ/d) was correlated with plasma Se concentration of this population with an r value of 0.27 (P=0.0003, two-tailed) (Figure 6-2 A). However, this correlation was stronger if outlier values of Se intake (below 7.4 and above 22.2

ng/kJ/d) were discarded, resulting a correlation factor r = 0.33 (P < 0.0001). Neither dietary Se intake nor plasma Se status was correlated to platelet GPx activities.

Note: GPx activity data are shown in Chapter 5, section 5.4.4 & 5.4.6, n = 62.

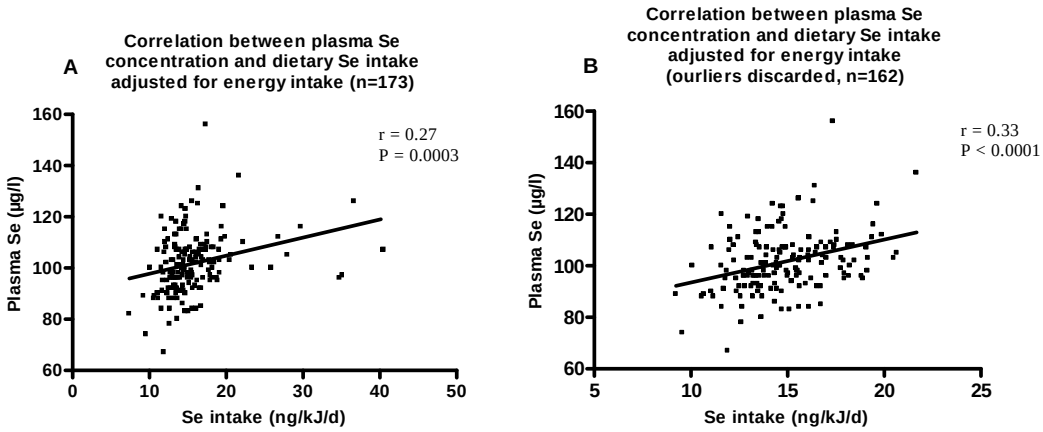


Figure 6-2 Correlation between plasma Se concentration and dietary Se intake.

6.4.3 Se intake/status and food sources (by CSIRO FFQ)

The food groups that made a major contribution (>5%) to the total daily Se intake in this population were the bread and cereals group (38.7%), the fish and seafood group (10.5%), the red meat and pork group (7.9%), dairy/soy products, (7.1%), nuts and seeds (7.0%) and processed meat (5.3%) (Table 6-1).

Plasma Se concentration was correlated significantly only with the food intake from the fish/ seafood group and the dairy group (both r = 0.33, P = 0.01, two-tailed) (Table 6-1). Cereal intake was correlated with plasma Se with a correlation factor of 0.22 which, however, did not achieve statistical significance (P = 0.09). These results were consistent with those found by using multiple regression models (Table 6-2), which showed that the strongest model was that which included only the fish/seafood group and the dairy/soy products group. This suggests that the variation of dietary intakes in these two food groups was the most important contributor to observed differences in plasma Se in the cohort.

Table 6-1 Se intake from food groups estimated using CSIRO FFQ and its correlation with plasma Se determined by Pearson's correlation test.

Food group	Se intake from food group (µg/d)	% Se intake from food group (%)	Correlation of Se intake from food groups with plasma Se	
			r	P value
Cereals, rice, pasta and breads	63.7	38.7	0.22	0.09
Fish, seafood	17.3	10.5	0.33	**0.01**
Red meat, pork	13.1	7.9	-0.14	0.30
Dairy, soy products	11.7	7.1	0.33	**0.01**
Nuts, seeds	11.5	7.0	0.07	0.59
Processed meat (sausages, ham, bacon)	8.7	5.3	0.02	0.90
Vegetables, vegetable juice, chips	7.51	4.56	0.06	0.63
Chicken	6.17	3.75	0.01	0.92
Savoury dishes, soup, stews	6.00	3.64	0.02	0.89
Cakes and biscuits		2.96	0.04	0.75
Eggs	4.61	2.8	-0.03	0.81
Takeaway foods (except chips)	4.39	2.66	0.01	0.94
Fruit and fruit juice	2.31	1.4	-0.03	0.83
Spread, sauce, dressing, dips	1.00	0.61	0.08	0.54
Bars containing nuts and cereals	0.56	0.34	0.18	0.18
Crisps, confectionery	0.03	0.02	-0.21	0.11

P values in bold are statistically significant.

Table 6-2 Se intake from varous food groups and plasma Se concentrations analysed using multiple regression models

Multiple regression model	Food groups*	Beta value	P value
Model I All food groups included Adjusted R^2 = 0.067; F(20,39) = 1.2, P < 0.297	Fish, seafood	0.49	**0.01**
Model II Protein-rich groups included** Adjusted R^2 = 0.104; F(7,52) = 1.98, P < 0.076	Fish, seafood	0.34	**0.01**
	Dairy, soy products	0.24	0.07
Model III Only top 2 predictors from model II included Adjusted R^2 = 0.124 F(2,57) = 5.19 p < **0.008**	Fish, seafood	0.29	**0.02**
	Dairy, soy products	0.26	**0.04**

P values in bold are statistically significant. * Only food group with a P value < 0.05 is shown. ** food groups included in this model were cereals, rice, pasta and bread group, red meat and pork group, dairy and soy products group, nuts and seeds group, chicken, eggs and fish/seafood group.

6.4.4 Correlation between Se intake, plasma Se and the intake of other nutrients measured using CSIRO FFQ and ACCV FFQ

The estimated daily intake of Se of the study population was positively correlated with the intake of the majority of nutrients captured by CSIRO FFQ with r ranging from 0.15 to 0.70 (P at the 0.05 or 0.01 level, two-tailed) as shown in Table 6-3. The intake of total nicotinic acid and total protein had the strongest correlation to Se intake (r = 0.72 and r = 0.70 respectively, P at the 0.01 level, two-tailed). Among all the nutrients analysed, the correlation between the intake of Vitamin C or retinol and the intake of Se was the weakest, although still significant (r = 0.18 and r = 0.15, respectively, P at the 0.05 level, two tailed). Plasma Se concentration was positively correlated with the percentage energy from protein (r = 0.28, P at the 0.01 level, two tailed) and negatively correlated with alcohol intake (r = - 0.15, P = 0.05) (Table 6-3). However, correlations may occur by chance due to multiple testing.

Similarly, the daily intake of Se was correlated with the dietary intake of all of the nutrients captured by the ACCV FFQ (r values ranging from 0.20 to 0.57, P at either 0.05 or 0.01 level, two-tailed) except that of alcohol, as shown in Table 6-4. Plasma Se concentration was not correlated with the intake of any of the nutrients estimated by the ACCV FFQ.

Table 6-3 Correlation between daily intake of Se or plasma Se concentration and other nutrients intakes based on the CSIRO FFQ results (n=173)

Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]
Water	0.51**	-0.47	Magnesium	0.69**	0.10	Total nicotinic acid	0.72**	0.11	Monounsaturated fat	0.63**	-0.05
Total sugar	0.41**	-0.19	Phosphorus	0.65**	0.08	Vitamin C	0.18*	0.06	Polyunsaturated fat	0.55**	-0.05
Complex CHO	0.64**	0.56	Iron	0.64**	0.08	Vitamin E	0.54**	-0.04	% energy from protein	0.13	0.28**
Fibre	0.57**	0.13	Copper	0.54**	0.09	Vitamin B_6	0.56**	0.11	% energy from CHO	-0.08	0.08
Nitrogen	0.70**	0.10	Zinc	0.64**	0.05	Vitamin B_{12}	0.34**	0.06	% energy from complex CHO	00.10	0.13
Kjoules	0.68**	-0.02	Retinol	0.15*	-0.01	Free folate	0.38**	0.10	% energy from sugar	-0.14	-0.01
Protein	0.70**	0.09	Carotene	0.44**	0.09	Total folate	0.47**	0.12	% energy from fat	0.21**	-0.13
Total fat	0.62**	-0.06	Total vitamin A	0.23**	0.01	Pantothenate	0.63**	0.08	% energy from poly. Fat	0.18*	-0.06
Total CHO	0.61**	0.02	Thiamin	0.60**	0.07	Biotin	0.55**	0.06	% energy from mono. Fat	0.22**	-0.10
Sodium	0.64**	0.03	Riboflavin	0.54**	0.11	Alcohol	-0.06	-0.15*	% energy from sat. fat	0.07	-0.12
Potassium	0.60**	0.07	Nicotinic acid	0.68**	0.13	Cholesterol	0.53**	0.04	% energy from alcohol	-0.21**	-0.11
Calcium	0.39**	0.03	Pot. Nicotinic acid	0.70**	0.08	Saturated fat	0.50**	-0.06			

[a] Correlation to daily intake of Se; [b] Correlation to plasma Se concentration, as determined by Pearson's correlation test.

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level, 2-tailed.

Table 6-4 Correlation between daily intake of Se or plasma Se concentration and other nutrient intakes based on the ACCV FFQ results as (n=165)

Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]	Nutrient	r[a]	r[b]
Kjoules	0.57**	0.01	Sugars	0.38**	0.004	Folate	0.38**	0.11	Retinol	0.30**	-0.10
Total fat	0.55**	-0.42	Starch	0.51**	0.06	Iron	0.44**	0.09	Riboflavin	0.39**	0.11
Saturated fat	0.48**	-0.08	Fibre	0.37**	0.13	Magnesium	0.47**	-0.07	Sodium	0.51**	0.04
Polyunsaturated fat	0.48**	0.01	Alcohol	0.001	-0.12	Nicotinic acid	0.45**	0.07	Thiamine	0.43**	0.12
Monounsaturated fat	0.56**	-0.03	Carotene	0.31**	0.08	Niacin equivalent	0.48**	0.06	Vitamin C	0.20*	-0.01
Protein	0.49**	0.04	Calcium	0.39**	0.09	Phosphorus	0.51**	0.09	Vitamin E	0.47**	0.01
CHO	0.51**	0.04	Cholesterol	0.36**	0.002	Potassium	0.46**	0.06	Zinc	0.51**	0.06

[a] Correlation to daily intake of Se; [b] Correlation to plasma Se concentration, as determined by Pearson's correlation test.

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level, 2-tailed.

6.5 Discussion

Plasma Se concentration of the study population (mean 102 µg/l) was found to be almost identical to that of another group of Adelaide residents reported in a recent survey study (Lyons et al., 2004) and being in the middle of the literature-based Australian reference range of plasma Se concentration i.e. 77 – 122 µg/l (Daniels et al., 2000; Lyons et al., 2004; Lyons et al., 2005; Jacobson et al., 2006; Jacobson et al., 2007; Lymbury et al., 2008). However, the plasma Se concentrations reported here are lower compared with the data from the USA, where Se concentration is mainly high in cereal-growing regions (Reilly, 2004). Survey studies showed that USA population had mean plasma Se concentrations ranging from 126 to 160 µg/l (Bleys et al., 2008; Kiremidjian-Schumacher et al., 1994; Nomura et al., 2000). Using plasma Se concentration of 70 µg/l as the cut-off level for optimal plasma GPx activity (Alfthan et al., 1991; Levander et al., 1983), our study cohort may be considered as replete in Se. However, it has been shown that higher plasma Se concentrations (100-200 µg/l) were required for maximization of other GPxs (Neve, 1995; Ravn-Haren et al., 2007), such as platelet GPx and thrombocyte GPx. The nil correlation between plasma Se concentration and platelet GPx activity in this study suggests that platelet GPx activity may have reached a plateau at a plasma Se concentration of 100 µg/l or above, which limits its usage as a biomarker of Se status in Se-replete populations. Nevertheless, the clinical significance of maximizing these selenoproteins is unknown. There is increasing evidence suggesting that a plasma Se concentration of 120 µg/l or greater may be required for reducing cancer risk (Combs, 2005; Duffield-Lillico et al., 2003).

The estimated dietary intake of Se for this group of older healthy men was 164 µg/d, which is much higher than the current recommended daily intake (RDI) of Se for men (70 µg/d) (National Health and Medical Research Council, 2005) and being well above that of most of the countries except Canada, America and Venezuela (Combs, 2001; Rayman, 2005). Previous studies showed mean plasma concentrations were 90 µg/l and 65.6 µg/l in populations with average Se intake of 48 µg/d and 41.6 µg/d, respectively (Reilly et al., 1990; Sunde et al., 2008). Therefore, this high level of intake is unexpected given the plasma Se concentration was only 102 µg/l in this population. Moreover, this level of intake almost doubled the figure reported in previous Australian studies, which was 87-89 µg/d for men (Thomson, 2004). Although FFQ surveys often overestimate nutrient intake, the Se intake of this study cohort was found to be even greater than that of the US middle-aged men (153 µg/d) as reported by Zhou et al.

(Zhou et al., 2003). The amount of Se in food is related to soil concentrations (Reilly, 1996). Se levels in the US foods, such as bread and meat, were reported to be higher than those of Australia (Morris and Levander, 1970; Tinggi et al., 1992). Therefore, the dietary Se intake data in the present study should be interpreted with caution as a US food composition database was used for the calculation of Se intake. It is possible that Se in Australian foods may actually be lower than that of foods in the USA. If such data were available and used in the composition database, the estimated daily intake of Se may be lower than the currently reported one. Inaccuracies in Se composition data may explain the low correlation factor ($r = 0.33$) between Se intake and plasma Se concentration in our study relative to previous studies showing stronger correlations ($r = 0.52 - 0.87$) between these two parameters (Reilly et al., 1990; Sunde et al., 2008).

The major food sources of Se (>5%) identified in this study were bread/cereals (39% of dietary Se intake) and other protein-rich foods (37% of dietary Se intake), such as fish, meat, diary, processed meat, nuts, chicken and eggs. This is consistent with the findings of previous studies that bread and cereal foods are one of the most important dietary sources of Se in the Western diet although their actual Se concentration is moderate (Barrett et al., 1989; Borawska et al., 2004; Gonzalez et al., 2006; Reilly et al., 1990). Protein foods are important dietary sources of Se as they are major components in human diet and high in selenoamino acids (McNaughton and Marks, 2002; Tinggi et al., 1992). It has been shown that protein-rich foods contributed to more than 60% of dietary Se intake in children and in elderly people (Gonzalez et al., 2006; Reilly et al., 1990). This is also supported by the significant correlation found between plasma Se concentration and the percentage of energy intake from protein in this study. Plasma Se concentration was correlated to the consumption of fish/seafood and dairy/soy products in our study, suggesting higher bioavailabilities of Se from these foods. This is in agreement with earlier studies, which showed fish consumption had a significant impact on plasma/serum Se concentrations (Arnaud et al., 2006; Bates et al., 2007; Hagmar et al., 1998; Huang et al., 1995). Nuts, particularly Brazil nuts, are rich in Se (Cotton et al., 2004). They can be a good source of dietary Se, however, the amount of consumption is usually small in the Australian diet and could explain the nil correlation with plasma Se.

In conclusion, this group of older men living in Adelaide had an adequate Se intake and appeared to be replete in Se status for maximizing plasma concentration and platelet GPx activities. Although based on the fluorimetric measure of plasma Se, 93% of the

men in this study had plasma Se concentration below 120 µg/l, which is considered optimal for cancer prevention (Combs et al., 2001). The CSIRO FFQ was shown to be a useful tool in terms of estimating dietary Se intake in this population. However, the weak correlation of Se intake with plasma Se concentration highlighted the need to establish a local food composition database of Se for a more reliable dietary assessment. Important dietary sources of Se found in this population were bread/cereals and specific protein-rich foods (fish/seafood and dairy/soy products). The hypothesis that bioavailability of Se in these latter foods may be higher than that in other food groups should be tested in controlled trials.

6.6 References

ACCV, A.-C. C. V. (2003) ACCV Dietary Questionnaire. Victoria, Anti-Cancer Council Victoria.

ALFTHAN, G., ARO, A., ARVILOMMI, H. & HUTTUNEN, J. (1991) Selenium metabolism and platelet glutathione peroxidase activity in healthy Finnish men: effects of selenium yeast, selenite, and selenate. *American Journal of Clinical Nutrition,* 53**,** 120-125.

ARNAUD, J., BERTRAIS, S., ROUSSEL, A. M., ARNAULT, N., RUFFIEUX, D., FAVIER, A., BERTHELIN, S., ESTAQUIO, C., GALAN, P., CZERNICHOW, S. & HERCBERG, S. (2006) Serum selenium determinants in French adults: the SU.VI.M.AX study. *British Journal of Nutrition,* 95**,** 313-320.

BAGHURST, K. I. & BAGHURST, P. (1981) The measurement of usual dietary intake in individuals and groups. *Trans Menz Found,* 3**,** 139-160.

BAGHURST, K. I., CRAWFORD, D., WORSLEY, A., SYRETTE, J. A., RECORD, S. J. & BAGHURST, P. A. (1988a) The Victorian Nutrition Survey: a profile of the energy, macronutrient and sodium intakes of the population. *Community Health Studies,* 12**,** 42-54.

BAGHURST, K. I., CRAWFORD, D. A., WORSLEY, A. & RECORD, S. J. (1988b) The Victorian Nutrition Survey--intakes and sources of dietary fats and cholesterol in the Victorian population. *Medical Journal of Australia,* 149**,** 12-15, 18-20.

BAGHURST, K. I. & RECORD, S. (1983) Intake and sources in selected Australian subpopulations of dietary constituents implicated in the etiology of chronic diseases. *Journal of Food and Nutrition,* 40**,** 1-15.

BAGHURST, K. I. & RECORD, S. J. (1984) A computerised dietary analysis system for use with diet diaries or food frequency questionnaires. *Community Health Studies,* 8**,** 11-18.

BALL, K., MISHRA, G., THANE, C. & HODGE, A. (2003) How well do Australian women comply with dietary guidelines? *Public Health Nutrition,* 7**,** 443-452.

BARRETT, J., PATTERSON, C., REILLY, C. & TINGGI, U. (1989) *Selenium in the diet of children with phenylketonuria,* London, Royal Society of Chemistry.

BATES, C. J., PRENTICE, A., BIRCH, M. C. & DELVES, H. T. (2007) Dependence of blood indices of selenium and mercury on estimated fish intake in a national survey of British adults. *Public Health Nutrition,* 10**,** 508-517.

BLEYS, J., NAVAS-ACIEN, A. & GUALLAR, E. (2008) Serum selenium levels and all-cause, cancer, and cardiovascular mortality among US adults. *Archives of Internal Medicine,* 168**,** 404-410.

BORAWSKA, M. H., WITKOWSKA, A. M., HUKALOWICZ, K. & MARKIEWICZ, R. (2004) Influence of dietary habits on serum selenium concentration. *Annals of Nutrition and Metabolism,* 48**,** 134-140.

CHAN, W., BROWN, J. & BUSS, D. (1994) *Miscellaneous foods. Supplement to McCance and Widdowson's the composition fo foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

COMBS, G., JR. (2005) Current evidence and research needs to support a health claim for selenium and cancer prevention. *Journal of Nutrition,* 135**,** 343-347.

COMBS, G. F., JR. (2001) Selenium in global food systems. *British Journal of Nutrition,* 85**,** 517-547.

COMBS, G. F., JR., CLARK, L. C. & TURNBULL, B. W. (2001) An analysis of cancer prevention by selenium. *Biofactors,* 14**,** 153-159.

COTTON, P. A., SUBAR, A. F., FRIDAY, J. E. & COOK, A. (2004) Dietary sources of nutrients among US adults, 1994 to 1996. *Journal of the American Dietetic Association,* 104**,** 921-930.

DANIELS, L. A., GIBSON, R. A. & SIMMER, K. N. (2000) Indicators of selenium status in Australian infants. *Journal of Paediatrics and Child Health,* 36**,** 370-374.

DUFFIELD-LILLICO, A. J., DALKIN, B. L., REID, M. E., TURNBULL, B. W., SLATE, E. H., JACOBS, E. T., MARSHALL, J. R. & CLARK, L. C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU International,* 91**,** 608-612.

ETMINAN, M., FITZGERALD, J. M., GLEAVE, M. & CHAMBERS, K. (2005) Intake of selenium in the prevention of prostate cancer: a systematic review and meta-analysis. *Cancer Causes and Control,* 16**,** 1125-1131.

GEBHARDT, S. E., PEHRSSON, P. R., CUTRUFELLI, R. L., LEMAR, L. E., HOWE, J. C., HAYTOWITZ, D. B., NICKLE, M. S., HOLCOMB, G. T., SHOWELL, B. A., THOMAS, R. G., EXLER, J. & HOLDEN, J. M. (2007) USDA National Nutrient Database for Standard Reference.

GONZALEZ, S., HUERTA, J. M., FERNANDEZ, S., PATTERSON, E. M. & LASHERAS, C. (2006) Food intake and serum selenium concentration in elderly people. *Annals of Nutrition and Metabolism,* 50**,** 126-131.

GROMER, S., EUBEL, J. K., LEE, B. L. & JACOB, J. (2005) Human selenoproteins at a glance. *Cellular and Molecular Life Sciences,* 62**,** 2414-2437.

HAGMAR, L., PERSSON-MOSCHOS, M., AKESSON, B. & SCHUTZ, A. (1998) Plasma levels of selenium, selenoprotein P and glutathione peroxidase and their correlations to fish intake and serum levels of thyrotropin and thyroid hormones: a study on Latvian fish consumers. *European Journal of Clinical Nutrition,* 52**,** 796-800.

HATFIELD, D. L., CARLSON, B. A., XU, X. M., MIX, H. & GLADYSHEV, V. N. (2006) Selenocysteine incorporation machinery and the role of selenoproteins in development and health. *Progress in Nucleic Acid Research and Molecular Biology,* 81**,** 97-142.

HODGE, A., PATTERSON, A. J., BROWN, W. J., IRELAND, P. & GILES, G. (2000) The Anti Cancer Council of Victoria FFQ: relative validity of nutrient intakes compared with weighed food records in young to middle-aged women in a study of iron supplementation. *Australian and New Zealand Journal of Public Health,* 24**,** 576-583.

HOLLAND, B., BROWN, J. & BUSS, D. (1993) *Fish and fish products. The third supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., UNWIN, I. & BUSS, D. (1991a) *Vegetables, herbs and spices. The fifth supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., UNWIN, I. & BUSS, D. (1992a) *Fruit and nuts. The first supplement to McCance and Widdowson's the composition of foods* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., WELCH, A. & BUSS, D. (1992b) *Vegetable dishes. The second supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., WELCH, A., UNWIN, I., BUSS, D., PAUL, A. & SOUTHGATE, D. (1991b) *McCance and Widdowson's the composition of foods* London, The Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food.

HUANG, W., AKESSON, B., SVENSSON, B. G., SCHUTZ, A., BURK, R. F. & SKERFVING, S. (1995) Selenoprotein P and glutathione peroxidase (EC 1.11.1.9) in plasma as indices of selenium status in relation to the intake of fish. *British Journal of Nutrition,* 73**,** 455-461.

IRELAND, P., JOLLEY, P., GILES, G., O'DEA, K., POWLES, J., RUTISHAUSER, I., WAHLQVIST, M. & WILLIAMS, J. (1994) Development of the Melbourne FFQ: a food frequency questionnaire for use in an Australian prospective study involving an ethnically diverse cohort. *Asia Pacific Journal of Clinical Nutrition,* 3**,** 19-31.

JACOBSON, G. A., NARKOWICZ, C., TONG, Y. C. & PETERSON, G. M. (2006) Plasma glutathione peroxidase by ELISA and relationship to selenium level. *Clinica Chimica Acta,* 369**,** 100-103.

JACOBSON, G. A., TONG, Y. C., TOWNSEND, A. T., FEATHERSTONE, A. M., BALL, M., ROBERTSON, I. K. & PETERSON, G. M. (2007) Selenium status in Southern Tasmania. *European Journal of Clinical Nutrition,* 61**,** 1057-1063.

KIREMIDJIAN-SCHUMACHER, L., ROY, M., WISHE, H., COHEN, M. & STOTZKY, G. (1994) Supplementation with selenium and human immune cell functions. II. Effect on cytotoxic lymphocytes and natural killer cells. *Biological Trace Element Research,* 41**,** 115-127.

KOH, T.-S. & BENSON, T. (1983) Critical re-appraisal of fluorometric method for determination of selenium in biological materials. *Analytical Chemistry,* 66**,** 918-926.

LAZARUS, R., WILSON, A., GLIKSMAN, M. & AIKEN, J. (1995) Repeatability of nutrient intakes estimated by a semiquantitative food frequency questionnaire in elderly subjects. *Annals of Epidemiology,* 5**,** 65-68.

LEVANDER, O., ALFTHAN, G., ARVILOMMI, H., GREF, C., HUTTUNEN, J., KATAJA, M., KOIVISTOINEN, P. & PIKKARAINEN, J. (1983) Bioavailability of selenium to Finnish men as assessed by platelet glutathione peroxidase activity and other blood parameters. *American Journal of Clinical Nutrition,* 37**,** 887-897.

LYMBURY, R., TINGGI, U., GRIFFITHS, L., ROSENFELDT, F. & PERKINS, A. V. (2008) Selenium Status of the Australian Population: Effect of Age, Gender and Cardiovascular Disease. *Biological Trace Element Research.*

LYONS, G., JUDSON, G., ORTIZ-MONASTERIO, I., GENC, Y., STANGOULIS, J. & GRAHAM, R. (2005) Selenium in Australia: selenium status and biofortification of wheat for better health. *Journal of Trace Elements in Medicine and Biology,* 19**,** 75-82.

LYONS, G., JUDSON, G., STANGOULIS, J., PALMER, L., JONES, J. & GRAHAM, R. (2004) Trends in selenium status of South Australians. *Medical Journal of Australia,* 180**,** 383-386.

MARTIN, S. A., HAREN, M. T., MIDDLETON, S. M. & WITTERT, G. A. (2007) The Florey Adelaide Male Ageing Study (FAMAS): design, procedures & participants. *BMC Public Health,* 7**,** 126.

MCNAUGHTON, S. & MARKS, G. (2002) Selenium content of Australian foods: a review of literature values. *Journal of Food Compostion and Analysis,* 15**,** 169-182.

MINISTRY OF AGRICULTURE FISHERIES AND FOOD (1998) *Fatty acids. Supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

MORRIS, V. C. & LEVANDER, O. A. (1970) Selenium content of foods. *Journal of Nutrition,* 100**,** 1383-1388.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL, N. (2005) *Nutrition reference values for Australia and New Zealand,* Canberra, Australian government, Department of Health and Aging.

NEVE, J. (1995) Human selenium supplementation as assessed by changes in blood selenium concentration and glutathione peroxidase activity. *Journal of Trace Elements in Medicine and Biology,* 9**,** 65-73.

NOMURA, A. M., LEE, J., STEMMERMANN, G. N. & COMBS, G. F., JR. (2000) Serum selenium and subsequent risk of prostate cancer. *Cancer Epidemiology, Biomarkers and Prevention,* 9**,** 883-887.

RAVN-HAREN, G., BUGEL, S., KRATH, B. N., HOAC, T., STAGSTED, J., JORGENSEN, K., BRESSON, J. R., LARSEN, E. H. & DRAGSTED, L. O. (2007) A short-term intervention trial with selenate, selenium-enriched yeast and selenium-enriched milk: effects on oxidative defence regulation. *British Journal of Nutrition***,** 1-10.

RAYMAN, M. P. (2000) The importance of selenium to human health. *The Lancet,* 356**,** 233-241.

RAYMAN, M. P. (2005) Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society,* 64**,** 527-542.

REILLY, C. (1996) *Selenium in food and health,* London & New York, Blackie Academic & Professional.

REILLY, C. (Ed.) (2004) *The nutritional trace metals,* Oxford, Wiley-Blackwell.

REILLY, C., BARRETT, J. E., PATTERSON, C. M., TINGGI, U., LATHAM, S. L. & MARRINAN, A. (1990) Trace element nutrition status and dietary intake of children with phenylketonuria. *American Journal of Clinical Nutrition,* 52**,** 159-165.

ROHAN, T. E. & POTTER, J. D. (1984) Retrospective assessment of dietary intake. *American Journal of Epidemiology,* 120**,** 876-887.

ROHAN, T. E., RECORD, S. & COOK, M. G. (1987) Repeatability of estimates of nutrient and energy intake: the quantitative food frequency approach. *Nutrition Research,* 7**,** 125-137.

SUNDE, R. A., PATERSON, E., EVENSON, J. K., BARNES, K. M., LOVEGROVE, J. A. & GORDON, M. H. (2008) Longitudinal selenium status in healthy British adults: assessment using biochemical and molecular biomarkers. *British Journal of Nutrition,* 99 Suppl 3**,** S37-47.

THOMSON, C. D. (2004) Selenium and iodine intakes and status in New Zealand and Australia. *British Journal of Nutrition,* 91**,** 661-672.

TINGGI, U., REILLY, C. & PATERSON, C. (1992) Determination of selenium in foodstuffs using spectrofluorometry and hudride generation atomic absorption spectrometry. *Journal of Food Composition and Analysis,* 5**,** 269-280.

WOODS, R. K., STONEY, R. M., IRELAND, P. D., BAILEY, M. J., RAVEN, J. M., THIEN, F. C., WALTERS, E. H. & ABRAMSON, M. J. (2002) A valid food

frequency questionnaire for measuring dietary fish intake. *Asia Pacific Journal of Clinical Nutrition,* 11**,** 56-61.

ZHOU, B. F., STAMLER, J., DENNIS, B., MOAG-STAHLBERG, A., OKUDA, N., ROBERTSON, C., ZHAO, L., CHAN, Q. & ELLIOTT, P. (2003) Nutrient intakes of middle-aged men and women in China, Japan, United Kingdom, and United States in the late 1990s: the INTERMAP study. *Journal of Human Hypertension,* 17**,** 623-630.

7. Nutrient Intakes, Food Intakes, Plasma Mineral Concentrations and Correlation to DNA Damage in Lymphocytes as Measured by the Comet Assay in Older Healthy South Australian Men

7.1 Summary

The aims of this study were to evaluate the association between DNA damage in lymphocyte DNA measured by the alkaline Comet assay and 1) intake of food groups at baseline; 2) the dietary intake of nutrients at baseline and 3) plasma concentration of minerals using pooled intervention data in a group of older healthy South Australian men. The study was performed on 62 males (n = 62, age 56 ± 7.0 years, mean ± SD) who had completed all stages of the Se-biofortified wheat intervention study (see Chapter 5). The dietary intake of nutrients and food groups were determined using two food frequency questionnaires (FFQ), namely CSIRO FFQ and Anti-cancer Council Victoria (ACCV) FFQ. Plasma concentrations of Fe, Cu, Ca, Zn, Mg, Na, K, P and S, as well as DNA damage in lymphocytes were measured at four different stages of the intervention. The correlations between these variables were evaluated using Pearson's correlation test. Comet DNA damage in lymphocytes was correlated to 1) the intakes of the following food groups: red meat (r = -0.27; P at 0.05 level), take-away foods (r = -0.26; P at 0.05 level) and sauce/spread (r = -0.26; P at 0.05 level) and nuts/seeds (r = 0.50; P at 0.01 level); 2) the intakes of following macronutrients: complex carbohydrate (r = -0.26; P at 0.05 level), polyunsaturated fatty acids (PUFA) (r = 0.26; P at 0.05 level) and fibre (r = 0.29; P at 0.05 level) and 3) the intakes of following micronutrients: zinc (r = -0.30; P at 0.05 level), carotene (r = -0.27; P at 0.05 level), Se (r = 0.36; P at 0.01 level), Mg (r = 0.31; P at 0.05 level), riboflavin (r = 0.29; P at 0.05 level), nicotinic acid (r = 0.27; P at 0.05 level). Plasma S concentration was negatively correlated to lymphocyte DNA oxidation (r = -0.15; P at 0.05 level) while plasma K (r = 0.15; P at 0.05 level) and plasma Na (r = 0.13; P at 0.05 level) concentrations were positively correlated with DNA damage in lymphocytes. In conclusion, it appears that various food groups, macronutrients and micronutrients may have small but statistical significant effects on lymphocyte DNA damage measured by the alkaline Comet assay. The biological significance and direction of the observed trends needs to be tested in controlled trials.

7.2 Introduction

It is known that micronutrients play important roles in genome stability maintenance (Ames and Wakimoto, 2002; Fenech, 2007). A deficiency of micronutrients involved in genome maintenance, such as folate and vitamin B_{12}, has been shown to induce chromosomal instability, which is considered to be associated with an increase in risk of cancer (Beetstra et al., 2005; Fenech, 2001; Wang et al., 2006). Oxidative stress due to inadequate dietary antioxidant micronutrient intakes may result in damage to DNA. An increase in aberrant base lesions in DNA may be linked to mutation (Valko et al., 2007; Valko et al., 2006). It has been hypothesised that oxidatively modified DNA may play a role in human carcinogenesis, although the causal relationship is not yet conclusive. The Comet assay is a useful tool in measuring base damage and DNA strand breaks caused by environmental and dietary genotoxic stresses in human lymphocytes (Anderson and Plewa, 1998). As part of the Se-biofortified wheat intervention study (as described in Chapter 5), dietary intakes of nutrients (CSIRO FFQ and ACCV FFQ) and food groups (CSIRO FFQ) were surveyed at the beginning of the study using two food frequency questionnaires (FFQs). Plasma concentrations of biologically relevant minerals as well as Comet DNA damage in lymphocytes were measured at all stages of the study.

The hypotheses of this study are: 1) DNA damage is affected by micronutrient status and 2) DNA damage in human lymphocytes is associated with dietary intake of nutrients or food groups. Currently, there is limited knowledge on which specific nutrients, food groups or plasma mineral concentrations that are associated with basal endogenous DNA damage in human lymphocytes.

Therefore, the present study aimed to evaluate the association between the DNA damage in lymphocytes measured by the alkaline Comet assay and 1) the dietary intake of nutrients; 2) the intake of food groups and 3) plasma concentration of minerals in a group of older healthy South Australian men. The association of increased Se intake with alterations in plasma concentration of other minerals was also investigated.

7.3 Study design and methods

7.3.1 Subjects

All men were recruited from the Adelaide metropolitan and the Adelaide Hills areas of South Australia through local media advertisement (TV/radio/newspapers) from September to November 2004. Eligible participants were healthy males aged 40-70

years, not supplementing with selenium and not supplementing above-recommended daily intake (RDI) levels of folate, vitamin B_{12} and vitamin C. The following volunteers were excluded from the study: 1) cancer patients undergoing chemotherapy or radiotherapy; 2) persons sensitive to study foods, i.e. gluten/wheat intolerant; 3) those unable to comprehend or comply with the study protocol and 4) individuals not available for all sampling phases of the study. 179 volunteers were screened for Se concentration in plasma after an overnight fasting. Volunteers were required to complete two versions of food frequency questionnaires (FFQs) while they attended the clinic. The study was undertaken from February to August, 2005 and was approved by the Human Research Ethics Committee of the CSIRO Division of Human Nutrition and the University of Adelaide ethics committee.

7.3.2 Dietary intake instruments

Two dietary intake instruments or food frequency questionnaires (FFQ) were employed in this study to investigate the estimated dietary intake of nutrients in the study cohort, namely CSIRO Dietary Intake Questionnaire (Baghurst and Record, 1984) and the Anti-Cancer Council of Victoria (ACCV) Dietary Questionnaire (ACCV, 2003). Both of these dietary intake instruments were self-completed quantitative questionnaires. Simple instructions on completing the questionnaires were provided. The ACCV FFQ does not provide an estimate of Se intake but was used to identify those nutrients that associated significantly with plasma Se concentration. Only the CSIRO FFQ provides information on intake of food groups.

7.3.2.1 The CSIRO Food Frequency Questionnaire (CSIRO FFQ)

The CSIRO FFQ is a 20-page booklet including a list of over 180 food and beverage items together with qualitative and quantitative questions relating to food preparation practices and dietary habits (Baghurst and Baghurst, 1981). Participants were required to indicate how often a specified serving size of each food and beverage was usually consumed per month, week or day. Using the average daily consumptions of particular foods and the nutrient composition of the food item per unit weight taken from food tables, daily intakes of nutrients of an individual were calculated with the FREQUAN dietary analysis program (Baghurst and Record, 1984). The Australian NUTTAB95 nutrient database and British McCance and Widdowson's food composition tables were consulted as the main sources of information concerning the nutrient composition of foods (Chan et al., 1994; Holland et al., 1993; Holland et al., 1991a; Holland et al., 1992a; Holland et al., 1992b; Holland et al., 1991b; Ministry of Agriculture Fisheries

and Food, 1998). When these databases did not contain information on the food, or for particular nutrients not covered, such as Se content of Brazil nut, equivalent data were used from the food composition tables of the Department of Agriculture of US (Gebhardt et al., 2007).

This CSIRO FFQ was first developed in the 1980s and has been used extensively with Australian population samples in epidemiological and national survey work (Baghurst et al., 1988a; Baghurst et al., 1988b). This FFQ has been shown to have a high repeatability and consistency with other dietary intake measurement techniques, such as weighed food record and multiple repeat 24-h records, and demonstrated good reliability when correlated with urinary sodium and nitrogen measures (Baghurst and Record, 1983; Lazarus et al., 1995; Rohan and Potter, 1984; Rohan et al., 1987). It is regularly modified to remain updated with the trends in dietary habits. In the present study, one question regarding Brazil nut consumption was added to capture the Se intake from this important source of Se.

7.3.2.2 The Anti-Cancer Council Victoria Food Frequency Questionnaire (ACCV FFQ)

The four page format ACCV FFQ includes 74 food items with responses on a 10-point scale (1 = Never to 10 = 3 or more times per day) in the frequency section and detailed questions on specific foods over the previous 12 months (Ireland et al., 1994). A series of photographs was included to enable estimation of portion size. Also, 10 cross-check questions were used to adjust for overestimation of fruit and vegetable consumption. The completed ACCV FFQs are optically scanned and nutrient intakes are computed from FFQ responses using software developed by the ACCV based on the NUTTAB95 nutrient composition data (Ireland et al., 1994). Vitamin E and folate intake are computed using the British McCance and Widdowson's database. However, the analysis does not provide information on the estimated intakes of Se and Vit B_{12}.

The ACCV FFQ was initially developed in the late 1980s to measure dietary intake for use in an ethnically diverse cohort of Australian-born men and women (Ireland et al., 1994). The current ACCV FFQ has been validated against weighed food records and found to give reliable estimate of dietary intake (Hodge et al., 2000). It has been used in a number of large epidemiological studies in the Australia populations (Ball et al., 2003; Martin et al., 2007; Woods et al., 2002).

7.3.3 Determination of plasma mineral concentrations

Mineral nutrients (Fe, Cu, Ca, Zn, Mg, Na, K, P S and Se) in plasma were analysed during all stages of the intervention study in order to identify any effect of increasing Se status and to study correlation with DNA damage measured by the alkaline Comet assay. Whole blood collected (at baseline, 8, 16 and 24 week) in Li-heparin tubes from each volunteer was centrifuged at 2800 x g for 15 min at 4^{o}C. 500 µl of plasma was removed and kept at -80^{o}C until sent for analysis at Waite Analytical Services (WAS), a certified laboratory for mineral analysis. The concentrations of these minerals were analysed by Radial CIROS Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Spectro Analytical Instruments, Cleve, Germany), following digestion with acids. There was 1.9-3.0% variation from the standard reference (Seronorm Serum Lot JL4409) for each mineral. Measurements were done on single samples because no replicates of the samples from the trial were available. The CVs of duplicate measures on the standard reference were 0.06-1.35% for these minerals in general.

7.3.4 DNA damage measured by the Comet assay

7.3.4.1 Comet assay

Lymphocyte DNA damage by Comet assay was measured using the alkaline method (Tice et al., 2000), which was modified for use with the high throughput CometSlide^{TM} HT (Trevigen Inc. Cat# 4252-02K-01). The assay was conducted on both fresh lymphocytes isolated on the visiting day (as described in Chapter 5.3.4.2) and on the same lymphocytes cryopreserved. In the latter case, cells of a participants from four visits were assayed in the same batch to eliminate inter-batch variance. Lymphocytes were thawed on ice and assayed directly after a cell count and viability count.

Cell suspension was combined with 1% low melting agarose gel solution in phosphate buffered saline (PBS, NaCl 8.0 g/l, KCl 0.2 g/l, KH_2PO_4, anhydrous 0.2 g/l, Na_2HPO_4, anhydrous 1.15g/l, pH adjusted to 7.5) which was kept in a 37 ^{o}C water bath, at a ratio of 1:10, then 75µl x 3 of the mixture was transferred to three of the sample spots on CometSlide HT (Trevigen). After agarose gel was set at 4^{o}C for 30 minutes, slides were immersed in 100ml lysis solution (100mmol/l EDTA disodium salt dehydrate, 2.5mol/l NaCl, 1% N-lauroylsarcosine, 10mmol/l Trizma base, pH adjusted to 10.0), in which 1% Triton X-100 and 10% DMSO was added on the day of experiment and chilled at 4^{o}C until use. The slides were incubated at 4^{o}C for 1 hour followed by two washes in MilliQ (18.2Ω resistivity) water for 5 minutes each. To produce single stranded DNA

and to express alkali labile sites (ALS) as single strand breaks, slides were incubated in ice-cold alkaline electrophoresis buffer (1 mmol/l EDTA, 300 mmol/l NaOH, pH adjusted to 13.0) for 25 minutes. Electrophoresis was conducted at 25 V, 450 mA for 20 minutes in the same alkaline buffer in a horizontal Comet assay electrophoresis tank with lid covered (Thistle Scientific; Power supply, Consort E 835). After electrophoresis, slides were washed three times in neutralization buffer (0.4 M Tris-HCl, pH adjusted to 7.5) for 5 minutes to neutralize the alkali in the gels. Slides were then immersed in 70% ethanol for 5 minutes, then air-dried at room temperature overnight. All procedures were performed under a dim light to avoid light-induced DNA damage. All chemicals were purchased from Sigma (Australia). A negative control (as described in Chapter 5.3.4.2) was included in each assay. After proper drying, slides were stained in propidium iodide solution (50 µg/ml in PBS) for 10 minutes before scoring and the staining procedure was repeated whenever the dye fluorescence faded.

7.3.4.2 Evaluation of DNA damage

The CometSlide^{TM} was scored visually using 20x objective of a fluorescence microscope (Nikon Eclipse E600 with tripleband filter; excitation wavelength 530 nm and emission wavelength 615 nm for far red) after staining in propidium iodide solution for 10 minutes in a dark room with dim light. Quantification was achieved by scoring 100 randomly selected comets per sample area (duplicate), assigning them to one of five DNA damage categories (refer Chapter 4 Figure 4.1), and converting the results to arbitrary units ranging from 0 to 400 (Collins, 2002; Tice et al., 2000). Calculation of arbitrary DNA damage units was based on the formula: DNA damage =Σήί x ί/4, where ήί is the number of cells in the damage degree category ί (0-4).

7.3.5 Statistical analysis

Data collected from individuals who had completed all stages of this study (n = 62) were included in the analysis. The differences between dietary intakes of nutrients measured by the two FFQs were compared using a paired samples t-test. All of the correlation relationships were determined by using two-tailed Pearson's correlation test. Because the CSIRO and ACCV FFQs were done only on baseline, correlation analysis with FFQ data was only done with parameters measured at baseline. In the case of correlation analysis of plasma minerals, correlation analysis with Comet DNA damage was done for pooled data of three treatment groups collected from each stage of the intervention trial. QQ-plots on standard residues of all plasma mineral concentrations

were performed to test the normality of the data sets. For the investigation on the effects of increased Se intake during the intervention on plasma mineral concentration, the significance of effect of treatment and time for each mineral was measured using general linear model repeated measures mixed between-within subjects ANOVA (Pallant, 2005) on the delta value of each follow-up time point (i.e. baseline value subtracted from the actual value measured at that time-point) with baseline values included as covariates to take account of effect of the baseline value on the delta value. Differences with P value <0.05 were considered to be statistically significant. Statistical analyses were performed using the statistical package SPSS for WINDOWS (version 16.0, SPSS Inc, Chicago).

7.4 Results

7.4.1 Estimated intake of nutrients and food groups measured using two FFQs

The estimated dietary intakes of nutrient measured using CSIRO FFQ and ACCV FFQ at the beginning of the study are presented in Table 7-1. Mean and SD were calculated based on dietary intakes of subjects who had completed the study with valid dietary data available. The CSIRO FFQ and the ACCV FFQ dietary intake values were in good agreement except for fibre, sugar, sodium, potassium, calcium, magnesium, retinol, carotene, vitamin C and vitamin E, which were significantly higher and for nicotinic acid, total folate and alcohol, which were significantly lower in the CSIRO FFQ. The estimated intake of various food groups measured using CSIRO FFQ are presented in Table 7-2.

Table 7-1 Estimated daily intake of nutrients measured using CSIRO FFQ (n=60) and ACCV FFQ (n=57) at the beginning of the study.

Nutrient	CSIRO FFQ	ACCV FFQ	t-test P value	RDI (for men, 50-70yr)
Energy (Kjoules/d)	10884 ± 2580	10216 ± 2991	0.11	-
Total CHO (g/d)	308 ± 77	255 ± 81	**0.0001**	-
Complex CHO / Starch (g/d)	134 ± 40	138 ± 49	0.43	-
Total sugar (g/d)	172 ± 55	115 ± 43	**0.0001**	-
Fibre (g/d)	31 ± 10	27 ± 9	**0.008**	30
Protein (g/d)	106 ± 29	108 ± 41	0.72	64
Nitrogen (g/d)	17 ± 5	-	-	-
Total fat (g/d)	97 ± 29	96 ± 31	0.96	-
Cholesterol (mg/d)	311 ± 123	331 ± 148	0.27	-
Saturated fat (g/d)	40 ± 14	38 ± 14	0.60	-
Monounsaturated fat (g/d)	35 ± 11	34 ± 11	0.99	-
Polyunsaturated fat (g/d)	16 ± 6	15 ± 6	0.74	-
Sodium (mg/d)	3662 ± 981	3131 ± 1150	**0.003**	-
Potassium (mg/d)	4611 ± 1193	3439 ± 1007	**0.0001**	-
Calcium (mg/d)	1233 ± 571	1031 ± 364	**0.02**	1000
Magnesium (mg/d)	417 ± 117	376 ± 121	**0.02**	420
Phosphorus (mg/d)	1790 ± 556	1846 ± 598	0.47	1000
Iron (mg/d)	16 ± 5	16 ± 7	0.97	8
Zinc (mg/d)	14 ± 4	14 ± 4	0.98	14
Copper (mg/d)	2 ± 1	-	-	-
Selenium (µg/d)	158 ± 56	-	-	70
Retinol (µg/d)	722 ± 828	445 ± 168	**0.01**	-
Carotene (µg/d)	4541± 1493	2859 ± 1018	**0.0001**	-
Total vitamin A (µg/d)	1479 ± 873	-	-	900
Thiamin (mg/d)	2 ± 1	2 ± 1	0.08	1.2
Riboflavin (mg/d)	3 ± 1	3 ± 1	0.57	1.3
Nicotinic acid (mg/d)	24 ± 7	28 ± 12	**0.02**	-
Pot. Nicotinic acid (mg/d)	20 ± 6	-	-	-
Total nicotinic acid (mg/d)	44 ± 12	48 ± 20	0.08	16
Vitamin C (mg/d)	196 ± 134	156 ± 82	**0.02**	45
Vitamin E (mg/d)	11 ± 4	9 ± 3	**0.001**	-
Vitamin B_6 (mg/d)	2 ± 1	-	-	1.7
Vitamin B_{12} (µg/d)	5 ± 4	-	-	2.4
Free folate (µg/d)	172 ± 76	-	-	-
Total folate (µg/d)	285 ± 106	335 ± 122	**0.01**	400
Pantothenate (mg/d)	6 ± 2	-	-	-
Biotin (µg/d)	26 ± 9	-	-	-
Alcohol (g/d)	12 ± 13	20 ± 22	**0.01**	-

P values in bold are statistically significant. Missing data are due to lack of intake measure calculation in the ACCV FFQ.

Table 7-2 Estimated intake of various food groups measured using CSIRO FFQ at the beginning of the study (n = 60)*.

Food group	Daily Intake (g)	
(n = 60)	Mean	SD
Cereals, rice, pasta and breads	258	99
Fish, seafood	32	23
Red meat, pork	52	31
Dairy, soy products	489	365
Nuts, seeds	4	5
Processed meat (sausages, ham, bacon)	36	26
Vegetables, vegetable juice, chips	312	115
Chicken	32	24
Savoury dishes, soup, stews	104	55
Cakes and biscuits	40	44
Eggs	16	14
Takeaway foods (except chips)	39	34
Fruit and fruit juice	436	284
Sauce, spread, dressing, dips,	36	17
Bars containing nuts and cereals	7	10
Crisps, confectionery	5	7

* Food group data are not provided by ACCV FFQ.

7.4.2 Impact of Se-fortified wheat intervention on plasma mineral concentrations

The concentrations of plasma minerals by study group and time of visit are shown in Table 7-3. Plasma concentrations of the majority of the minerals (Fe, Cu, P, Na, Zn, Ca and Mg) remained unchanged during the study. There was a significant treatment group effect observed in plasma K concentrations (mixed between-within subjects ANOVA $P < 0.05$ for treatment). A one-way ANOVA test showed that the difference in plasma K concentrations between intervention groups existed since baseline and persisted on to later stages of the study ($P< 0.05$, data not shown) indicating that the observed differences were not due to increased intake of Se-fortified wheat. There was a time effect seen in plasma sulphur (S) concentrations (mixed between-within subjects ANOVA $P < 0.05$ for time) which increased slightly from February (summer) to August (winter).

Table 7-3 Plasma mineral concentrations (mean ± SD) of the study subjects at baseline and after week 8, 16, and 24 weeks (WK).

Study group	Baseline	8 WK	16 WK	24 WK	ANOVA P
		Plasma iron (Fe) (mg/l)			
CONTROL N=22	1.2 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	1.3 ± 0.4	0.41 for time
BIOFORT N=19	1.3 ± 0.5	1.4 ± 0.4	1.3 ± 0.4	1.5 ± 0.6	
PROFORT N=21	1.2 ± 0.3	1.2 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	
ANOVA P		0.18 for treatment			
		Plasma copper (Cu) (mg/l)			
CONTROL N=22	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	0.43 for time
BIOFORT N=19	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	
PROFORT N=21	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	
ANOVA P		0.14 for treatment			
		Plasma phosphorus (P) (mg/l)			
CONTROL N=22	125.9 ± 13.7	131.0 ± 14.8	131 ± 16.0	129.8 ± 14.4	0.50 for time
BIOFORT N=19	125.3 ± 11.6	129.1 ± 14.4	128.7 ± 10.9	137.4 ± 11.5	
PROFORT N=21	127.3 ± 16.5	131 ± 17.1	133.5 ± 14.0	132.5 ± 13.7	
ANOVA P		0.81 for treatment			
		Plasma sulphur (S) (mg/l)			
CONTROL N=22	1110 ± 61	1152 ± 79	1150 ± 55	1170 ± 69	**0.02** for time
BIOFORT N=19	1148 ± 71	1155 ± 75	1156 ± 60	1213 ± 70	
PROFORT N=21	1114 ± 56	1138 ± 60	1158 ± 50	1167 ± 60	
ANOVA P		0.93 for treatment			
		Plasma sodium (Na) (mg/l)			
CONTROL N=22	3177 ± 81	3223 ± 61	3282 ± 50	3282 ± 80	0.55 for time
BIOFORT N=19	3158 ± 96	3237 ± 60	3263 ± 68	3353 ± 61	
PROFORT N=21	3167 ± 86	3224 ± 70	3262 ± 80	3295 ± 97	
ANOVA P		0.17 for treatment			
		Plasma potassium (K) (mg/l)			
CONTROL N=22	160.0 ± 13.7	164.6 ± 7.7	168.4 ± 10.9	162.5 ± 10.2	0.62 for time
BIOFORT N=19	150.5 ± 11.3	155.0 ± 8.2	159.2 ± 10.8	160.4 ± 7.1	
PROFORT N=21	156.0 ± 8.0	160.5 ± 6.8	161.1 ± 9.3	161.3 ± 8.9	
ANOVA P		**<0.05** for treatment			
		Plasma zinc (Zn) (mg/l)			
CONTROL N=22	0.89 ± 0.09	0.92 ± 0.12	0.91 ± 0.15	0.90 ± 0.07	0.98 for time
BIOFORT N=19	0.90 ± 0.12	0.87 ± 0.08	0.86 ± 0.08	0.94 ± 0.10	
PROFORT N=21	0.86 ± 0.11	0.86 ± 0.09	0.91 ± 0.07	0.89 ± 0.07	
ANOVA P		0.58 for treatment			
		Plasma calcium (Ca) (mg/l)			
CONTROL N=22	93.5 ± 2.7	96.4 ± 3.1	97.6 ± 2.5	97.4 ± 2.7	0.81 for time
BIOFORT N=19	94.9 ± 3.3	96.7 ± 4.8	97.9 ± 4.2	100.9 ± 3.3	
PROFORT N=21	94.5 ± 3.8	96.4 ± 3.1	98.2 ± 2.2	98.9 ± 2.7	
ANOVA P		0.41 for treatment			
		Plasma magnesium (Mg) (mg/l)			
CONTROL N=22	19.9 ± 0.9	20.2 ± 1.4	20.4 ± 1.4	20.9 ± 1.1	0.30 for time
BIOFORT N=19	19.9 ± 1.4	20.4 ± 1.3	20.7 ± 1.2	21.2 ± 1.1	
PROFORT N=21	19.4 ± 0.9	20.2 ± 1.4	20.8 ± 1.2	20.8 ± 1.2	
ANOVA P		0.14 for treatment			

Baseline differences were adjusted by subtracting the baseline value from all data collected from the same individual using mixed between-within subjects ANOVA. P value in bold is statistically significant.

7.4.3 Correlation between intake of minerals and plasma mineral concentrations at baseline

The correlations between dietary intakes of mineral Fe, Cu, Ca, Mg, Na, K, Zn, and P and their plasma concentrations were evaluated as shown in Table 7-4. The baseline plasma concentration of K was positively correlated with its dietary intake with an r value of 0.28 (P at 0.05 level) and the baseline plasma concentrations of Cu and Fe were negatively correlated with their dietary intake (r = -0.3, P at 0.05 level). However, these significant relationships were only observed with the CSIRO FFQ and may occur due to multiple testing.

Table 7-4 Correlation coefficient (r values) between plasma concentrations and dietary intakes of minerals determined by Pearson's correlation test

FFQ	Iron	Copper	Calcium	Magnesium	Sodium	Potassium	Zinc	Phosphorus
CSIRO	-0.3*****	-0.3*****	-0.11	-0.11	-0.03	0.26*****	0.14	-0.1
ACCV	-0.25	-	-0.15	0.10	0.1	0.06	0.13	-

* Correlation is significant at the 0.05 level (2-tailed). Missing data are due to lack of intake measures calculation in the ACCV FFQ. Note: Neither of the FFQ measures the dietary intake of sulphur.

7.4.4 Correlation between intake of nutrients or food groups and DNA damage in lymphocytes measured by using Comet assay at baseline

Correlations between baseline DNA damage in lymphocytes and dietary intake of nutrients are presented in Table 7-5 but only results for nutrients with significant effects are shown. Dietary intake of zinc, complex carbohydrate and carotene were negatively correlated to DNA damage in lymphocytes, while dietary intake of selenium, PUFA, fibre, magnesium and nicotinic acid were positively correlated to DNA damage in lymphocytes. However, these correlations did not exist consistently in fresh and in cryopreserved cells, or in different DNA damage categories, or for the same nutrient measured by different dietary assessment tools.

Table 7-6 presents the correlations between lymphocyte DNA damage and intake of various food groups. Baseline Comet DNA damage was negatively correlated with the intake of red meat (excluding processed meat, sausages, ham and bacon), take-away foods (except chips) and spreads/sauces. There was a strong positive correlation between nuts/seeds consumption and Comet DNA damage of all categories (r = 0.50, P = 0.01), but only for the Comet assay data with fresh lymphocytes.

Note: The actual data of baseline DNA damage in both fresh and cryopreserved lymphocytes measured by the Comet assay can be found in Table 5.8a&b and Table 5.9a&b in Chapter 5.

Table 7-5 Correlation coefficient (r value) between baseline DNA damage in lymphocytes and dietary intake of various nutrients determined by Pearson's correlation test

Nutrient	Comet DNA damage in fresh lymphocytes			Comet DNA damage in cryopreserved lymphocytes		
	Cat. 0-3	Cat. 4	Cat. 0-4	Cat. 0-3	Cat. 4	Cat. 0-4
ACCV FFQ						
Zinc	-0.04	0.003	-0.02	-0.33*	-0.19	-0.30*
Polyunsaturated fat	0.21	0.27*	0.26*	-0.05	0.08	0.01
CSIRO FFQ						
Selenium	0.26*	0.41**	0.36**	-0.21	-0.04	-0.15
Complex carbohydrate	0.04	0.08	0.07	-0.26*	0.18	-0.15
Fibre	0.07	0.29*	0.19	-0.03	-0.13	-0.09
Magnesium	0.13	0.31*	0.24	-0.01	-0.13	-0.07
Carotene	-0.27*	-0.06	-0.19	-0.12	-0.07	-0.11
Riboflavin	0.14	0.29*	0.23	-0.02	-0.13	-0.08
Nicotinic acid	0.14	0.27*	0.22	-0.2	-0.01	-0.13

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Table 7-6 Correlation coefficient (r value) between baseline DNA damage in lymphocytes measured by the Comet assay and dietary intake of various food groups (CSIRO FFQ) determined by Pearson's correlation test

Food group	Comet DNA damage in fresh lymphocytes			Comet DNA damage in cryopreserved lymphocytes		
	Cat. 0-3	Cat. 4	Cat. 0-4	Cat. 0-3	Cat. 4	Cat. 0-4
Red meat	-0.19	-0.13	-0.17	-0.27*	-0.18	-0.27*
Take-away foods (except chips)	-0.16	-0.09	-0.14	-0.24	-0.20	-0.26*
Sauces, spread, dressing, dips	0.07	-0.02	0.03	-0.26*	-0.04	-0.19
Nuts, seeds	0.31*	0.62**	0.50**	0.14	0.18	0.18

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

7.4.5 Correlation between plasma mineral concentrations and DNA damage in lymphocytes using pooled data from the intervention

The correlations between DNA damage in lymphocytes as measured by the Comet assay and the plasma concentrations of all the minerals tested are presented in Table 7-7. Increased plasma S concentration was associated with decreased category 0-3 and category 4 DNA damage measured in fresh lymphocyte samples (r = - 0.21 P = 0.01 and

r = - 0.15 P = 0.05, 2-tailed). Weak positive correlations between category 4 DNA damage and plasma Na or K were also observed (r = 0.13 – 0.15 P = 0.05, 2-tailed).

Table 7-7 Correlation coefficient (r value) between the plasma concentration of minerals and the DNA damage in lymphocytes measured by using the Comet assay, pooled data of four visits (n=248).

Plasma Mineral	Comet DNA damage in fresh lymphocytes			Comet DNA damage in cryopreserved lymphocytes		
	Cat. 0-3	Cat. 4	Cat. 0-4	Cat. 0-3	Cat. 4	Cat. 0-4
Iron	-0.09	-0.08	-0.09	0.03	-0.06	-0.02
Copper	-0.04	0.03	0.003	-0.02	-0.11	-0.07
Phosphorus	-0.02	0.02	<0.001	-0.03	0.02	-0.003
Sulphur	-0.21**	-0.08	-0.15*	-0.01	0.01	-0.003
Sodium	0.01	0.13*	0.09	0.01	0.11	0.06
Potassium	0.05	0.15*	0.11	0.09	0.14*	0.12
Zinc	-0.08	-0.02	-0.05	-0.03	-0.09	-0.06
Calcium	-0.09	0.09	0.02	-0.02	0.06	0.02
Magnesium	-0.03	0.05	0.02	0.001	0.04	0.02

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

Pooled data, all data collected from three treatment groups at all stages of the study

7.5 Discussion

The intakes of nutrients and food groups reported in this study were similar to those reported in a previous study performed in another group of South Australian residents (Fenech et al., 2005). The dietary intake results indicate that our study cohort had micronutrient intakes equivalent or above the RDI level of intakes for most of the micronutrients, except for folate, retinol and biotin.

It was found that dietary intake of Zn was negatively correlated with baseline DNA damage in cryopreserved lymphocyte. This is consistent with the important role of Zn as an essential component of many proteins and transcription factors involved in DNA damage response and repair (Dreosti, 2001). It has been shown that Zn deficiency increased DNA single strand breaks (Comet assay) in human and rat cells (Ho and Ames, 2002; Ho et al., 2003; Yan et al., 2008). Inadequate intake of Zn may compromise DNA integrity by impairing the function of zinc-containing proteins, such as zinc-containing DNA-repair proteins p53 and apurinic endonuclease. These results show that red meat intake was also negatively correlated to the Comet DNA damage in our study. This could be explained by the fact that red meat is a good dietary source of Zn, which contributed up to 14% of Zn intake in the our study population. However,

another study did not find such a correlation between red meat intake and Comet DNA damage in a Mediterranean population (Giovannelli et al., 2002).

This study showed that dietary carotene was negatively correlated to category 0-3 Comet DNA damage but not to total DNA damage in this study. However, Watters and colleagues reported no association between dietary carotene or plasma concentration of this antioxidant and Comet DNA damage in lymphocytes of healthy American adults (Watters et al., 2007). The intake of sauce and spread group was also negatively correlated to oxidative DNA damage in this study. However, it is not clear which nutrients in this food group are accountable for this association. There is one study showing that increased consumption of tomato sauce based entrée reduced oxidative damage in prostate cancer patients, which was possibly related to the carotenoid content in tomato sauce (Chen et al., 2001).

The findings of this study indicate that higher intake of dietary Se could lead to greater Comet DNA damage of all categories in this group of healthy men. Increased serum Se, however, was reported to be associated with decreased Comet DNA damage in a European population (Dusinska et al., 2003). This may be due to the baseline Se status (mean serum Se 88 µg/l) of that study population being much lower than that of the current study, and also being below the optimal level for effective selenoprotein functions. Se is an essential trace element, being a component of more than 20 selenoproteins, such as glutathione peroxidase (GPx) (Kryukov et al., 2003). Lymphocytes deficient in Se and vitamin E were found to be more susceptible to oxidative DNA damage *in vitro* (Chuang and Hu, 2005). However, metabolites of Se compounds such as hydrogen selenide and methyl selenol can generate superoxide ions, resulting in increased oxidative stress and oxidative DNA damage (Spallholz et al., 2001; Tarze et al., 2007). In animal studies, a U-shaped dose-response relationship has been demonstrated between Se intake or status and oxidative DNA damage in lymphocytes as measured by the Comet assay (Waters et al., 2003; Waters et al., 2005). High dietary intake of Se as selenite increased the concentration of 8-hydroxy-2'-deoxyguanosine in rat liver DNA (Wycherly et al., 2004). Therefore, increasing intake of Se in individuals who are Se-replete may prove to be harmful rather than beneficial to DNA damage. This however is countered by the fact that the intervention with Se process-fortified or Se bio-fortified wheat in this cohort did not cause an increase in Comet DNA damage (see Chapter 5) and that the observed correlation effects may be

only coincidental or a chance effect given the multiple associations that were investigated.

The positive correlation of nuts/seeds intake and oxidative DNA damage was the strongest in this study, given that the actual amount of nuts/seeds consumption was only 4 g per day, and the effect was mainly attributable to Cat 4 DNA damage, which is likely to be indication of apoptotic or necrotic cell death (Fairbairn et al., 1995; Fairbairn and O'Neill, 1995; Fairbairn et al., 1996). Nuts are high in both monounsaturated fatty acids (MUFA) and PUFA (Gebhardt et al., 2007). A recent study showed that a single dose of MUFA-rich emulsion infusion given to healthy volunteers decreased cell proliferation and provoked necrotic cell death in lymphocytes (Cury-Boaventura et al., 2008). The results of this study also showed PUFA intake was positively correlated with DNA damage in lymphocytes. This may due to the fact that PUFA are more susceptible to peroxidation than monounsaturated fatty acids, which might induce cellular oxidative stress and increased DNA oxidation (Nenseter and Drevon, 1996). A study in women treated for breast cancer previously showed increased PUFA intake was associated with urinary oxidative damage biomarkers, indicating an increase in lipid peroxidation {Thomson, 2005 #974}. Another earlier study showed that Comet DNA damage was significantly increased after consumption of a 15% PUFA diet compared to a 5% PUFA diet in young healthy males, when vitamin E was not supplemented (Jenkinson et al., 1999). This study population had 5% of total energy intake from PUFA. Another possible explanation is the mixed nuts/seeds are rich in Se, particularly Brazil nuts. However, Se concentration in Brazil nuts varied widely depending on soil Se concentration and pH (Chang et al., 1995). It is also possible that the DNA damage is induced by other toxic metals, such as barium and radium, co-accumulated by the plant (Parekh et al., 2008) or due to mycotoxins such as aflatoxin, but a recent study showed that the Comet assay in lymphocytes is not sensitive to aflatoxin exposure in humans (Anderson et al., 1999). A recent study showed that Brazil nuts were as effective in improving plasma Se concentration and GPx activity as selenomethionine tablets in a group of New Zealanders (Thomson et al., 2008). Increased intake of nuts may be associated with reduced risk of cardiovascular disease and diabetes (Allen, 2008), however, the effect of increasing nuts/seeds intake on DNA integrity should also be considered when making dietary recommendations in a Se-replete population.

There is evidence suggesting that deficiency in Mg, riboflavin or nicotinic acid was associated with an increased oxidative stress and DNA damage in *in vitro* studies (Ganji et al., 2008; Jia et al., 2008; Manthey et al., 2006; Martin et al., 2003; Martin et al., 2008; Tang et al., 2008; Zhang et al., 2008). Dietary fibre supplementation appears to be inhibiting oxidative stress in cardiac tissue of rats (Diniz et al., 2003). However, results from this current study showed that at about RDI level of intake, dietary Mg, riboflavin, nicotinic acid as well as fibre were positively correlated to category 4 Comet DNA damage, which is likely to be indicative of apoptotic or necrotic cell death (Fairbairn et al., 1995; Fairbairn and O'Neill, 1995; Fairbairn et al., 1996). The negative correlation between take-away food intake and category 4 DNA damage in lymphocytes is hard to explain without further detailed investigation but might indicate inhibitory effects on the apoptotic or necrotic process.

The minerals measured in the present study were all within the expected range for a healthy population (Fischbach and Dunning, 2004). Plasma S concentration was inversely associated with lymphocyte DNA damage. This might be related to some natural sulphur compounds present in human plasma which have antioxidative properties, such as glutathione and aminoethylcysteine ketimine decarboxylated dimer (Battin and Brumaghim, 2008; Marcon et al., 2003). There were positive and weak correlations between plasma K or Na concentrations and DNA damage in lymphocytes, relationships which need to be further investigated in future studies. Hyperosmolality in the form of elevated sodium chloride was shown to cause DNA double strand breaks in murine kidney cells as measured by the neutral Comet assay (Kultz and Chakravarty, 2001).

There are some limitations to this study. Firstly, the dietary intake of nutrients and food groups may not be accurate as they were estimated from the FFQ survey. Secondly, the plasma mineral concentrations are not necessarily reliable biomarkers for body status of minerals because they do not necessarily reflect mineral status within lymphocytes in which DNA damage was measured (Hess et al., 2007). They are either tightly regulated by homeostatic mechanisms and show relatively low variation (reduced absorption and/or increased excretion), such as Ca, or reflect recent intake rather than long-term status with potential for interactions between minerals occurring (Krajcovicova-Kudlackova et al., 2003; Leung and Li, 1993). Some discrepancy between the findings of the two FFQs and the frequent lack of associations between estimated nutrient intakes and plasma levels were observed in this study. In a relatively small group of

homogenous samples with similar dietary patterns, it is likely that narrow range in plasma mineral concentrations make the correlations between Comet DNA damage and plasma mineral concentrations undetectable. Thirdly, multiple comparisons are likely to give a few false significant correlations due to chance and it is probable that application of a Bonferroni correction to the analysis would render most of the observed correlations to relative insignificance. Therefore, some of the correlations detected in this study were relatively weak and should be interpreted with caution. However, this study still provides some preliminary data regarding the possible effect of nutrient/food intake and plasma mineral concentration on DNA damage of human lymphocytes and inform future studies on the expected variation in the parameters measured and likely strength of association between Comet assay DNA damage and micronutrient intake or status.

7.6 References

ACCV, A.-C. C. V. (2003) ACCV Dietary Questionnaire. Victoria, Anti-Cancer Council Victoria.

ALLEN, L. H. (2008) Priority areas for research on the intake, composition, and health effects of tree nuts and peanuts. *Journal of Nutrition,* 138**,** 1763S-1765S.

ANDERSON, D. & PLEWA, M. J. (1998) The International Comet Assay Workshop. *Mutagenesis,* 13**,** 67-73.

ANDERSON, D., YU, T. W., HAMBLY, R. J., MENDY, M. & WILD, C. P. (1999) Aflatoxin exposure and DNA damage in the comet assay in individuals from the Gambia, West Africa. *Teratogenesis, Carcinogenesis, and Mutagenesis,* 19**,** 147-155.

BAGHURST, K. I. & BAGHURST, P. (1981) The measurement of usual dietary intake in individuals and groups. *Trans Menz Found,* 3**,** 139-160.

BAGHURST, K. I., CRAWFORD, D., WORSLEY, A., SYRETTE, J. A., RECORD, S. J. & BAGHURST, P. A. (1988a) The Victorian Nutrition Survey: a profile of the energy, macronutrient and sodium intakes of the population. *Community Health Studies,* 12**,** 42-54.

BAGHURST, K. I., CRAWFORD, D. A., WORSLEY, A. & RECORD, S. J. (1988b) The Victorian Nutrition Survey--intakes and sources of dietary fats and cholesterol in the Victorian population. *Medical Journal of Australia,* 149**,** 12-15, 18-20.

BAGHURST, K. I. & RECORD, S. (1983) Intake and sources in selected Australian subpopulations of dietary constituents implicated in the etiology of chronic diseases. *Journal of Food and Nutrition,* 40**,** 1-15.

BAGHURST, K. I. & RECORD, S. J. (1984) A computerised dietary analysis system for use with diet diaries or food frequency questionnaires. *Community Health Studies,* 8**,** 11-18.

BALL, K., MISHRA, G., THANE, C. & HODGE, A. (2003) How well do Australian women comply with dietary guidelines? *Public Health Nutrition,* 7**,** 443-452.

BATTIN, E. E. & BRUMAGHIM, J. L. (2008) Metal specificity in DNA damage prevention by sulfur antioxidants. *Journal of Inorganic Biochemistry,* 102**,** 2036-2042.

CHAN, W., BROWN, J. & BUSS, D. (1994) *Miscellaneous foods. Supplement to McCance and Widdowson's the composition fo foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

CHANG, J. C., GUTENMANN, W. H., REID, C. M. & LISK, D. J. (1995) Selenium content of Brazil nuts from two geographic locations in Brazil. *Chemosphere,* 30**,** 801-802.

CHEN, L., STACEWICZ-SAPUNTZAKIS, M., DUNCAN, C., SHARIFI, R., GHOSH, L., VAN BREEMEN, R., ASHTON, D. & BOWEN, P. E. (2001) Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. *Journal of the National Cancer Institute,* 93**,** 1872-1879.

CHUANG, C. H. & HU, M. L. (2005) Effect of dietary vitamin E and selenium on DNA damage in fresh and frozen tissues. *International Journal for Vitamin and Nutrition Research,* 75**,** 251-256.

COLLINS, A. (2002) The Comet Assay - principles, applications, and limitations. IN DIDENKO, V. V. (Ed.) *Methods in molecular biology, in situ detection of DNA damage, methods and protocols.* Totowa, New Jersey, Humana Press.

CURY-BOAVENTURA, M. F., GORJAO, R., DE LIMA, T. M., FIAMONCINI, J., TORRES, R. P., MANCINI-FILHO, J., SORIANO, F. G. & CURI, R. (2008) Effect of olive oil-based emulsion on human lymphocyte and neutrophil death. *JPEN. Journal of Parenteral and Enteral Nutrition,* 32**,** 81-87.

DINIZ, Y. S., CICOGNA, A. C., PADOVANI, C. R., SILVA, M. D., FAINE, L. A., GALHARDI, C. M., RODRIGUES, H. G. & NOVELLI, E. L. (2003) Dietary restriction and fibre supplementation: oxidative stress and metabolic shifting for cardiac health. *Canadian Journal of Physiology and Pharmacology,* 81**,** 1042-1048.

DREOSTI, I. E. (2001) Zinc and the gene. *Mutation Research,* 475**,** 161-167.

DUSINSKA, M., KAZIMIROVA, A., BARANCOKOVA, M., BENO, M., SMOLKOVA, B., HORSKA, A., RASLOVA, K., WSOLOVA, L. & COLLINS, A. R. (2003) Nutritional supplementation with antioxidants decreases chromosomal damage in humans. *Mutagenesis,* 18**,** 371-376.

FAIRBAIRN, D. W. & O'NEILL, K. L. (1995) Necrotic DNA degradation mimics apoptotic nucleosomal fragmentation comet tail length. *In Vitro Cellular and Developmental Biology. Animal,* 31**,** 171-173.

FAIRBAIRN, D. W., OLIVE, P. L. & O'NEILL, K. L. (1995) The comet assay: a comprehensive review. *Mutation Research,* 339**,** 37-59.

FAIRBAIRN, D. W., WALBURGER, D. K., FAIRBAIRN, J. J. & O'NEILL, K. L. (1996) Key morphologic changes and DNA strand breaks in human lymphoid cells: discriminating apoptosis from necrosis. *Scanning,* 18**,** 407-416.

FENECH, M., BAGHURST, P., LUDERER, W., TURNER, J., RECORD, S., CEPPI, M. & BONASSI, S. (2005) Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability--results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis,* 26**,** 991-999.

FISCHBACH, F. & DUNNING, M. (2004) *A Manual of Laboratory and Diagnostic Tests,* Philadelphia, Lippincott Williams & Wilkins.

GANJI, S. H., QIN, S., ZHANG, L., KAMANNA, V. S. & KASHYAP, M. L. (2008) Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. *Atherosclerosis*.

GEBHARDT, S. E., PEHRSSON, P. R., CUTRUFELLI, R. L., LEMAR, L. E., HOWE, J. C., HAYTOWITZ, D. B., NICKLE, M. S., HOLCOMB, G. T., SHOWELL,

B. A., THOMAS, R. G., EXLER, J. & HOLDEN, J. M. (2007) USDA National Nutrient Database for Standard Reference.

GIOVANNELLI, L., SAIEVA, C., MASALA, G., TESTA, G., SALVINI, S., PITOZZI, V., RIBOLI, E., DOLARA, P. & PALLI, D. (2002) Nutritional and lifestyle determinants of DNA oxidative damage: a study in a Mediterranean population. *Carcinogenesis,* 23**,** 1483-1489.

HESS, S. Y., PEERSON, J. M., KING, J. C. & BROWN, K. H. (2007) Use of serum zinc concentration as an indicator of population zinc status. *Food and Nutrition Bulletin,* 28**,** S403-429.

HO, E. & AMES, B. N. (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 16770-16775.

HO, E., COURTEMANCHE, C. & AMES, B. N. (2003) Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *Journal of Nutrition,* 133**,** 2543-2548.

HODGE, A., PATTERSON, A. J., BROWN, W. J., IRELAND, P. & GILES, G. (2000) The Anti Cancer Council of Victoria FFQ: relative validity of nutrient intakes compared with weighed food records in young to middle-aged women in a study of iron supplementation. *Australian and New Zealand Journal of Public Health,* 24**,** 576-583.

HOLLAND, B., BROWN, J. & BUSS, D. (1993) *Fish and fish products. The third supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., UNWIN, I. & BUSS, D. (1991a) *Vegetables, herbs and spices. The fifth supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., UNWIN, I. & BUSS, D. (1992a) *Fruit and nuts. The first supplement to McCance and Widdowson's the composition of foods* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., WELCH, A. & BUSS, D. (1992b) *Vegetable dishes. The second supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

HOLLAND, B., WELCH, A., UNWIN, I., BUSS, D., PAUL, A. & SOUTHGATE, D. (1991b) *McCance and Widdowson's the composition of foods* London, The Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food.

IRELAND, P., JOLLEY, P., GILES, G., O'DEA, K., POWLES, J., RUTISHAUSER, I., WAHLQVIST, M. & WILLIAMS, J. (1994) Development of the Melbourne FFQ: a food frequency questionnaire for use in an Australian prospective study involving an ethnically diverse cohort. *Asia Pacific Journal of Clinical Nutrition,* 3**,** 19-31.

JENKINSON, A. M., COLLINS, A. R., DUTHIE, S. J., WAHLE, K. W. & DUTHIE, G. G. (1999) The effect of increased intakes of polyunsaturated fatty acids and vitamin E on DNA damage in human lymphocytes. *FASEB Journal,* 13**,** 2138-2142.

JIA, H., LI, X., GAO, H., FENG, Z., ZHAO, L., JIA, X., ZHANG, H. & LIU, J. (2008) High doses of nicotinamide prevent oxidative mitochondrial dysfunction in a cellular model and improve motor deficit in a Drosophila model of Parkinson's disease. *Journal of Neuroscience Research,* 86**,** 2083-2090.

KRAJCOVICOVA-KUDLACKOVA, M., URSINYOVA, M., BLAZICEK, P., SPUSTOVA, V., GINTER, E., HLADIKOVA, V. & KLVANOVA, J. (2003)

Free radical disease prevention and nutrition. *Bratislavske Lekarske Listy,* 104**,** 64-68.

KRYUKOV, G. V., CASTELLANO, S., NOVOSELOV, S. V., LOBANOV, A. V., ZEHTAB, O., GUIGO, R. & GLADYSHEV, V. N. (2003) Characterization of mammalian selenoproteomes. *Science,* 300**,** 1439-1443.

KULTZ, D. & CHAKRAVARTY, D. (2001) Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 1999-2004.

LAZARUS, R., WILSON, A., GLIKSMAN, M. & AIKEN, J. (1995) Repeatability of nutrient intakes estimated by a semiquantitative food frequency questionnaire in elderly subjects. *Annals of Epidemiology,* 5**,** 65-68.

LEUNG, P. L. & LI, X. L. (1993) The changes of metabolism balance of zinc and copper in gastric juice with widely varying dietary zinc intake. *Biological Trace Element Research,* 39**,** 33-39.

MANTHEY, K. C., RODRIGUEZ-MELENDEZ, R., HOI, J. T. & ZEMPLENI, J. (2006) Riboflavin deficiency causes protein and DNA damage in HepG2 cells, triggering arrest in G1 phase of the cell cycle. *Journal of Nutritional Biochemistry,* 17**,** 250-256.

MARCON, F., ANDREOLI, C., ROSSI, S., VERDINA, A., GALATI, R. & CREBELLI, R. (2003) Assessment of individual sensitivity to ionizing radiation and DNA repair efficiency in a healthy population. *Mutation Research,* 541**,** 1-8.

MARTIN, H., RICHERT, L. & BERTHELOT, A. (2003) Magnesium deficiency induces apoptosis in primary cultures of rat hepatocytes. *Journal of Nutrition,* 133**,** 2505-2511.

MARTIN, H., URING-LAMBERT, B., ADRIAN, M., LAHLOU, A., BONET, A., DEMOUGEOT, C., DEVAUX, S., LAURANT, P., RICHERT, L. & BERTHELOT, A. (2008) Effects of long-term dietary intake of magnesium on oxidative stress, apoptosis and ageing in rat liver. *Magnesium Research,* 21**,** 124-130.

MARTIN, S. A., HAREN, M. T., MIDDLETON, S. M. & WITTERT, G. A. (2007) The Florey Adelaide Male Ageing Study (FAMAS): design, procedures & participants. *BMC Public Health,* 7**,** 126.

MINISTRY OF AGRICULTURE FISHERIES AND FOOD (1998) *Fatty acids. Supplement to McCance and Widdowson's the composition of foods,* London, The Royal Society of Chemistry and Ministry of Agriculture Fisheries and Food.

NENSETER, M. S. & DREVON, C. A. (1996) Dietary polyunsaturates and peroxidation of low density lipoprotein. *Current Opinion in Lipidology,* 7**,** 8-13.

PALLANT, J. (2005) *SPSS survival manual : a step by step guide to data analysis using SPSS for Windows (Version 12),* Crows Nest, N.S.W., Allen & Unwin.

PAREKH, P., KHAN, A., TORRES, M. & KITTO, M. (2008) Concentration of selenium, barium and radium in Brazil nuts. *Journal of Food Compostion and Analysis,* 2008**,** 332-335.

ROHAN, T. E. & POTTER, J. D. (1984) Retrospective assessment of dietary intake. *American Journal of Epidemiology,* 120**,** 876-887.

ROHAN, T. E., RECORD, S. & COOK, M. G. (1987) Repeatability of estimates of nutrient and energy intake: the quantitative food frequency approach. *Nutrition Research,* 7**,** 125-137.

SPALLHOLZ, J. E., SHRIVER, B. J. & REID, T. W. (2001) Dimethyldiselenide and methylseleninic acid generate superoxide in an in vitro chemiluminescence assay in the presence of glutathione: implications for the anticarcinogenic activity of L-selenomethionine and L-Se-methylselenocysteine. *Nutrition and Cancer,* 40**,** 34-41.

TANG, K., SHAM, H., HUI, E. & KIRKLAND, J. B. (2008) Niacin deficiency causes oxidative stress in rat bone marrow cells but not through decreased NADPH or glutathione status. *Journal of Nutritional Biochemistry,* 19**,** 746-753.

TARZE, A., DAUPLAIS, M., GRIGORAS, I., LAZARD, M., HA-DUONG, N. T., BARBIER, F., BLANQUET, S. & PLATEAU, P. (2007) Extracellular production of hydrogen selenide accounts for thiol-assisted toxicity of selenite against Saccharomyces cerevisiae. *Journal of Biological Chemistry,* 282**,** 8759-8767.

THOMSON, C. D., CHISHOLM, A., MCLACHLAN, S. K. & CAMPBELL, J. M. (2008) Brazil nuts: an effective way to improve selenium status. *American Journal of Clinical Nutrition,* 87**,** 379-384.

TICE, R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. & SASAKI, Y. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and Molecular Mutagenesis,* 35**,** 206-221.

VALKO, M., LEIBFRITZ, D., MONCOL, J., CRONIN, M. T., MAZUR, M. & TELSER, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology,* 39**,** 44-84.

VALKO, M., RHODES, C. J., MONCOL, J., IZAKOVIC, M. & MAZUR, M. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions,* 160**,** 1-40.

WATERS, D., SHEN, S., COOLEY, D., BOSTWICK, D., QIAN, J., COMBS, G., JR., GLICKMAN, L., OTEHAM, C., SCHLITTLER, D. & MORRIS, J. (2003) Effects of dietary selenium supplementation on DNA damage and apoptosis in canine prostate. *Journal of the National Cancer Institute,* 95**,** 237-241.

WATERS, D. J., SHEN, S., GLICKMAN, L. T., COOLEY, D. M., BOSTWICK, D. G., QIAN, J., COMBS, G. F., JR. & MORRIS, J. S. (2005) Prostate cancer risk and DNA damage: translational significance of selenium supplementation in a canine model. *Carcinogenesis,* 26**,** 1256-1262.

WATTERS, J. L., SATIA, J. A., KUPPER, L. L., SWENBERG, J. A., SCHROEDER, J. C. & SWITZER, B. R. (2007) Associations of antioxidant nutrients and oxidative DNA damage in healthy African-American and White adults. *Cancer Epidemiology, Biomarkers and Prevention,* 16**,** 1428-1436.

WOODS, R. K., STONEY, R. M., IRELAND, P. D., BAILEY, M. J., RAVEN, J. M., THIEN, F. C., WALTERS, E. H. & ABRAMSON, M. J. (2002) A valid food frequency questionnaire for measuring dietary fish intake. *Asia Pacific Journal of Clinical Nutrition,* 11**,** 56-61.

WYCHERLY, B. J., MOAK, M. A. & CHRISTENSEN, M. J. (2004) High dietary intake of sodium selenite induces oxidative DNA damage in rat liver. *Nutrition and Cancer,* 48**,** 78-83.

YAN, M., SONG, Y., WONG, C. P., HARDIN, K. & HO, E. (2008) Zinc deficiency alters DNA damage response genes in normal human prostate epithelial cells. *Journal of Nutrition,* 138**,** 667-673.

ZHANG, J., WANG, H., PENG, D. & TAYLOR, E. W. (2008) Further insight into the impact of sodium selenite on selenoenzymes: high-dose selenite enhances hepatic thioredoxin reductase 1 activity as a consequence of liver injury. *Toxicology Letters,* 176**,** 223-229.

8. Conclusions, Knowledge Gaps and Future Directions

8.1 Introduction

The essential role and the nutritional importance of selenium (Se) in biological systems has been well established. A recent study suggested that dietary Se supplementation may reduce risk for cancer, particularly prostate cancer (Clark et al., 1996). However, any observed beneficial effect of supplementation may depend on Se status and a supra-nutritional dose of Se or higher Se status may be required for the beneficial effect to occur, i.e. 200 µg/day as supplementation or a plasma Se concentration greater than 120 µg/l (Duffield-Lillico et al., 2002; Duffield-Lillico et al., 2003). Genome instability, resulting from accumulation of DNA damage, has been considered as the fundamental cause of certain degenerative diseases, such as cancer (Fenech, 2007). Increased DNA damage/genome instability has been used as a sensitive early biomarker to identify those individuals with increased cancer risk. Biomarkers of genome instability as measured by the cytokinesis-block micronucleus cytome (CBMN Cyt) assay have previously been shown to be predictive of cancer risks in humans (Bonassi et al., 2007; El-Zein et al., 2008; El-Zein et al., 2006). Selenium may protect DNA from oxidative damage through improved activity of selenoproteins, such as GPxs which act as free radical scavengers. However, the impact of Se status on genome stability is largely unknown and the safe dose-range of Se in humans is not clearly defined. The main focus of the present work was to (a) determine the optimal Se-met concentration for genome stability using an *in vitro* culture model and (b) investigate the bioavailability and bioefficacy of organic Se from biofortified or process-fortified wheat. These studies investigated the safe dose-range, optimal concentration for genome stability maintenance and resistance to gamma radiation and/or hydrogen peroxide induced genome damage *in vitro*, as well as measured plasma Se concentration, platelet glutathione peroxidase activity and DNA damage biomarkers *in vivo*. The intakes of nutrients, food groups and plasma mineral concentrations were measured by conducting survey studies and their correlations with Se intake/status and baseline lymphocyte DNA damage were also determined.

8.2 *In vitro* dose-response study for Se-met in human lymphocytes

The present *in vitro* study in long-term cultures of human lymphocytes clearly showed that Se-met is cytotoxic and genotoxic at high concentrations (> 340 µg/l or above) as indicated by inhibition of lymphocyte proliferation and increased frequencies of

apoptosis and necrosis. Se-met at concentrations ranging from 3 µg/l to 430 µg/l demonstrated no effect on i) total Comet DNA damage; ii) frequency of BN MNi and iii) gamma-irradiation or hydrogen peroxide induced DNA damage in long-term human lymphocyte cultures. Increasing the Se-met concentration to 430 µg/l seemed to slightly increase DNA base oxidation as measured by the Comet assay in combination with glycosylase treatment. Se-met concentration as low as 3 µg/l did not induce significantly greater DNA damage in this study. Although Se-met concentrations at the higher end showed clear evidence of cytotoxicity and genotoxicity, a clear U-shaped curved was not observed in the present study. However, significant trends for the reduction in both the frequencies of BN NPB and BN NBud as measured using the CBMN Cyt assay were evident. These two genome instability biomarkers have been shown to be associated with increased lung cancer risk in smokers (El-Zein et al., 2006). It appears that the CBMN Cyt assay is more sensitive to subtle beneficial effects of Se-met on genome stability than the Comet assay.

A few possible underlying mechanisms of toxicity include 1) excessive replacement of methionine by Se-met in proteins which may alter protein function; 2) superoxides generated by Se-met metabolites may increase cellular oxidative stress and resulting in increased oxidative DNA damage and 3) Se-met may affect polyamine metabolism and alter SeAM/SAM ratio and thereby interfere with many biological reactions, such as glutathione synthesis and DNA methylation. However, the mechanisms by which Se-met exerts its cytotoxicity and genotoxicity effects, as well as the exact cytotoxic and genotoxic dose of Se-met, between 430 µg/l and 1880 µg/l, are yet to be investigated in future studies.

The need to elucidate the mechanisms by which Se-met improve genome stability, as indicated by reduced frequencies of NPB and NBud is another clear direction for future studies. However, the apparent beneficial effects of Se-met on genome stability need to be verified in future studies using a more physiological culture medium. The currently used RPMI-1640 culture medium in i*n vitro* culture studies may not be ideal for testing the genotoxicity effect of Se-met or predicting effects of Se-met on genome stability *in vivo*. RPMI-1640 culture medium contains either below or above physiological levels of key nutrients that are involved in DNA repair, synthesis, methylation and are important for genome integrity maintenance, such as folate, methionine, riboflavin and zinc (Ames, 2001; Burtis et al., 2006; Crott et al., 2001; Fischbach and Dunning, 2004; Kimura et al., 2004). Differences in key nutrient components in RPMI relative to the

physiological environment *in vivo* may modify the activity of cells and their responses to oxidative stress (Leist et al., 1996). For example, human lung fibroblasts cultured in zinc-deficient medium had increased Comet DNA damage due to increased cellular oxidative stress due to zinc depletion in cells (Ho et al., 2003). It has been shown that methylenetetrahydrofolate reductase (MTHFR) activity can be modified by the high riboflavin concentrations in RPMI-1640 culture medium, masking the genotypic effect of this enzyme (Crott et al., 2001; Kimura et al., 2004).

8.3 *In vivo* Se supplementation study in healthy older South Australian men

The results from the intervention study (n = 62) suggests that increased Se intake up to 263 – 473 µg/d, including 105-315 µg/d from Se supplementation and 158 µg/d derived from habitual dietary intake, does not appear to have any adverse biological symptoms or genotoxic effect resulting from this short term intervention on this cohort of healthy men within the limit of the study time-frame. The higher end of this intake range was comparable to the upper level of intake of Se for adults in Australia (400 µg/d) as suggested by Australia's NHMRC {National Health and Medical Research Council, 2006 #287}; however, this was still much lower than the no observable adverse effect level (NOAEL) of 800 µg/d previously established as a result of earlier human studies (Food and Nutrition Board, 2000; Hathcock, 2004; Yang and Zhou, 1994). Se-met can be incorporated into proteins in place of methionine and its absorption could be limited by a methionine rich diet (Reilly, 1996). It has been suggested that at a nutritional level of intake (<300 µg/d), tissue Se level may reach a steady state due to protein turnover; therefore, long-term studies of accumulation of Se in body may be warranted if changes in protein turnover are expected to occur depending on background diet and disease state (Bost and Blouin, 2009; Schrauzer, 2000).

By the end of the intervention study, the mean plasma Se concentration reached a higher level in the BIOFORT group (193 µg/l) than in the PROFORT group (140 µg/l). This suggests that Se-methionine from biofortified wheat biscuits is more bioavailable and efficient in raising plasma Se concentrations in this Se-replete cohort. The purified Se-met used in the process-fortification seemed to be more sensitive to heat processing than the organic Se in the biofortified wheat resulting in oxidation and reduced bioavailability. Therefore, agronomic biofortification of wheat could be an economic and effective strategy in increasing dietary Se and Se status in humans. However, increased dietary intake of Se and/or plasma Se concentrations by supplementing with

biofortified or process fortified wheat did not modify the endogenous DNA damage in lymphocytes as measured by the Comet assay and/or the cellular antioxidant capacity as measured by the platelet GPx activity in the cohort studied. It is possible that the baseline Se status of individuals in this study cohort was already high enough to maximize platelet GPx activities and further Se supplementation did not alter the activities of this selenoenzyme and probably cancer risks in this group of males (Duffield-Lillico et al., 2002; Duffield-Lillico et al., 2003). These initial observations from this study are in agreement with results of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) and the European Prospective Investigation into Cancer and Nutrition (EPIC) study, which found that improved plasma Se concentrations were not associated with prostate cancer risk of healthy well-nourished US men (Allen et al., 2008; Lippman et al., 2009). Furthermore, increased Se concentration in plasma is not necessarily associated with increased Se concentration in lymphocytes, platelets or other organs. It has been demonstrated that one month Se supplementation with high-Se yeast increased plasma Se status but it was not correlated with Se concentration in prostate tissue in men (Gianduzzo et al., 2003). Given that no additional beneficial effect on either antioxidant capacity or genome stability effect of improved Se status was demonstrated in this study and the long-term toxicity is unknown, a cautious approach is required in recommending Se supplementation to avoid any toxic effects in Se-replete populations. Future Se supplementation studies conducted in populations with lower Se status or a higher Se requirement may provide more useful information, for example, prostate cancer patients (Brinkman et al., 2006), carriers of colon adenomas (Al-Taie et al., 2003), elderly people (Arnaud et al., 2009), heavy smokers (Peters et al., 2007) and some ethnic groups (Gill et al., 2009).

There is increasing evidence published after this part of the study was completed that the response to Se supplementation and the effect of Se status on cancer risk of an individual may depend on the genotype as a result of single nucleotide polymorphisms (SNPs) in some key genes, such as selenoprotein-P (*Sel P*), GPx-1 and selenoprotein 15 (*Sel 15*) (Arsova-Sarafinovska et al., 2008; Bermano et al., 2007; Cai et al., 2006; Foster et al., 2006; Hu et al., 2005; Hu and Diamond, 2003; Jablonska et al., 2008; Meplan et al., 2007). It has been shown that two different functional SNPs in *Sel P* gene were associated with differences in the concentration and activities of several selenoproteins, such as GPxs and thioredoxin reductase 1, as well as their responses to Se supplementation (Meplan et al., 2007). Ever-smokers with low Se status who had a Sel P genotype that is associated with lower plasma Sel P in men, and who were

homozygous for mitochondrial superoxide dismutase SNP Ala16Val were found to be at doubled risk of prostate cancer (Cooper et al., 2008). Among smokers, Jablonska *et al* found that those with *Sel 15* 1125 AA genotype may benefit most from a higher Se intake, whereas in those with GG or GA genotype, at Se concentration above 80 µg/l, the risk of developing lung cancer started to increase (Jablonska et al., 2008). The research areas of genome health nutrigenomics and nutrigenetics are relatively new, studying the interaction between nutrient intake/status and molecular events at genetic and cellular level, as well as the impact of genetic background on nutrient requirement and health outcome (Fenech, 2005; Fenech, 2008). Therefore, directions for future studies would include the identification of genetic subgroups that are more likely to benefit or are at an increased risk for toxic insults resulting from elevated Se status through biofortified wheat supplementation, as well as to determine the optimal level of Se intake for optimal genome stability and maintenance, possibly resulting in a reduction in individual cancer risk.

8.4 Se status, intake and food sources survey study in healthy older South Australian men

Data collected from the survey studies (n = 173) suggest that South Australian males had high Se intake (165 µg/d) from habitual diet alone; however, this level of intake almost double that reported previously in Australians. The mean plasma Se concentration (n = 179) of 102 µg/l, however, is almost identical to that reported in another recent study (Lyons et al., 2004). The correlation between Se intakes and plasma Se concentrations was lower than those reported in other studies. The accuracy of Se intake estimations may be limited by the non-local food composition database for Se employed in this study. Therefore the need to develop a local food composition table of Se for more accurate estimates is indicated.

The major dietary sources of Se identified in this study cohort were wheat and protein-rich foods, such as fish, meat and dairy products. The intakes of fish/seafood and dairy/soy products, particularly, are the food groups found to be associated with plasma Se concentration, suggesting higher bioavailability of Se in these foods. The relative bioavailability of Se from common food sources should be investigated in controlled trials.

8.5 References

AL-TAIE, O. H., SEUFERT, J., KARVAR, S., ADOLPH, C., MORK, H., SCHEURLEN, M., KOHRLE, J. & JAKOB, F. (2003) Selenium supplementation enhances low selenium levels and stimulates glutathione peroxidase activity in peripheral blood and distal colon mucosa in past and present carriers of colon adenomas. *Nutrition and Cancer,* 46**,** 125-130.

ALLEN, N. E., APPLEBY, P. N., RODDAM, A. W., TJONNELAND, A., JOHNSEN, N. F., OVERVAD, K., BOEING, H., WEIKERT, S., KAAKS, R., LINSEISEN, J., TRICHOPOULOU, A., MISIRLI, G., TRICHOPOULOS, D., SACERDOTE, C., GRIONI, S., PALLI, D., TUMINO, R., BUENO-DE-MESQUITA, H. B., KIEMENEY, L. A., BARRICARTE, A., LARRANAGA, N., SANCHEZ, M. J., AGUDO, A., TORMO, M. J., RODRIGUEZ, L., STATTIN, P., HALLMANS, G., BINGHAM, S., KHAW, K. T., SLIMANI, N., RINALDI, S., BOFFETTA, P., RIBOLI, E. & KEY, T. J. (2008) Plasma selenium concentration and prostate cancer risk: results from the European Prospective Investigation into Cancer and Nutrition (EPIC). *American Journal of Clinical Nutrition,* 88**,** 1567-1575.

AMES, B. N. (2001) DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutation Research,* 475**,** 7-20.

ARNAUD, J., AKBARALY, T. N., HININGER-FAVIER, I., BERR, C. & ROUSSEL, A. M. (2009) Fibrates but not statins increase plasma selenium in dyslipidemic aged patients - The EVA study. *Journal of Trace Elements in Medicine and Biology,* 23**,** 21-28.

ARSOVA-SARAFINOVSKA, Z., MATEVSKA, N., EKEN, A., PETROVSKI, D., BANEV, S., DZIKOVA, S., GEORGIEV, V., SIKOLE, A., ERDEM, O., SAYAL, A., AYDIN, A. & DIMOVSKI, A. J. (2008) Glutathione peroxidase 1 (GPX1) genetic polymorphism, erythrocyte GPX activity, and prostate cancer risk. *International Urology and Nephrology*.

BERMANO, G., PAGMANTIDIS, V., HOLLOWAY, N., KADRI, S., MOWAT, N. A., SHIEL, R. S., ARTHUR, J. R., MATHERS, J. C., DALY, A. K., BROOM, J. & HESKETH, J. E. (2007) Evidence that a polymorphism within the 3'UTR of glutathione peroxidase 4 is functional and is associated with susceptibility to colorectal cancer. *Genes and Nutrition,* 2**,** 225-232.

BONASSI, S., ZNAOR, A., CEPPI, M., LANDO, C., CHANG, W. P., HOLLAND, N., KIRSCH-VOLDERS, M., ZEIGER, E., BAN, S., BARALE, R., BIGATTI, M. P., BOLOGNESI, C., CEBULSKA-WASILEWSKA, A., FABIANOVA, E., FUCIC, A., HAGMAR, L., JOKSIC, G., MARTELLI, A., MIGLIORE, L., MIRKOVA, E., SCARFI, M. R., ZIJNO, A., NORPPA, H. & FENECH, M. (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis,* 28**,** 625-631.

BOST, M. & BLOUIN, E. (2009) Effect of supplementation with Se-enriched yeast and factors influencing Se concentration in plasma of transplant recipients. *Journal of Trace Elements in Medicine and Biology,* 23**,** 36-42.

BRINKMAN, M., REULEN, R. C., KELLEN, E., BUNTINX, F. & ZEEGERS, M. P. (2006) Are men with low selenium levels at increased risk of prostate cancer? *European Journal of Cancer,* 42**,** 2463-2471.

BURTIS, C., ASHWOOD, E. & BRUNS, D. (2006) *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics,* St. Louis, Elsevier Saunders.

CAI, L., MU, L. N., LU, H., LU, Q. Y., YOU, N. C., YU, S. Z., LE, A. D., ZHAO, J., ZHOU, X. F., MARSHALL, J., HEBER, D. & ZHANG, Z. F. (2006) Dietary selenium intake and genetic polymorphisms of the GSTP1 and p53 genes on the risk of esophageal squamous cell carcinoma. *Cancer Epidemiology, Biomarkers and Prevention,* 15**,** 294-300.

CLARK, L., COMBS, G., JR., TURNBULL, B., SLATE, E., CHALKER, D., CHOW, J., DAVIS, L., GLOVER, R., GRAHAM, G., GROSS, E., KRONGRAD, A., LESHER, J., JR., PARK, H., SANDERS, B., JR., SMITH, C. & TAYLOR, J. (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA,* 276**,** 1957-1963.

COOPER, M. L., ADAMI, H. O., GRONBERG, H., WIKLUND, F., GREEN, F. R. & RAYMAN, M. P. (2008) Interaction between single nucleotide polymorphisms in selenoprotein P and mitochondrial superoxide dismutase determines prostate cancer risk. *Cancer Research,* 68**,** 10171-10177.

CROTT, J. W., MASHIYAMA, S. T., AMES, B. N. & FENECH, M. (2001) The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. *Cancer Epidemiology, Biomarkers and Prevention,* 10**,** 1089-1096.

DUFFIELD-LILLICO, A., REID, M., TURNBULL, B., COMBS, G., JR., SLATE, E., FISCHBACH, L., MARSHALL, J. & CLARK, L. (2002) Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial. *Cancer Epidemiology, Biomarkers and Prevention,* 11**,** 630-639.

DUFFIELD-LILLICO, A. J., DALKIN, B. L., REID, M. E., TURNBULL, B. W., SLATE, E. H., JACOBS, E. T., MARSHALL, J. R. & CLARK, L. C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *BJU International,* 91**,** 608-612.

EL-ZEIN, R. A., FENECH, M., LOPEZ, M. S., SPITZ, M. R. & ETZEL, C. J. (2008) Cytokinesis-blocked micronucleus cytome assay biomarkers identify lung cancer cases amongst smokers. *Cancer Epidemiology, Biomarkers and Prevention,* 17**,** 1111-1119.

EL-ZEIN, R. A., SCHABATH, M. B., ETZEL, C. J., LOPEZ, M. S., FRANKLIN, J. D. & SPITZ, M. R. (2006) Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk. *Cancer Research,* 66**,** 6449-6456.

FENECH, M. (2005) The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. *Mutagenesis,* 20**,** 255-269.

FENECH, M. (2007) Nutrition and genome health. *Forum of Nutrition,* 60**,** 49-65.

FENECH, M. (2008) Genome health nutrigenomics and nutrigenetics--diagnosis and nutritional treatment of genome damage on an individual basis. *Food and Chemical Toxicology,* 46**,** 1365-1370.

FISCHBACH, F. & DUNNING, M. (2004) *A Manual of Laboratory and Diagnostic Tests,* Philadelphia, Lippincott Williams & Wilkins.

FOOD AND NUTRITION BOARD (2000) *Dietary reference intakes for vitamin C, vitamin E, selenium and carotenoids,* Washington DC, National Academy Press.

FOSTER, C. B., ASWATH, K., CHANOCK, S. J., MCKAY, H. F. & PETERS, U. (2006) Polymorphism analysis of six selenoprotein genes: support for a selective sweep at the glutathione peroxidase 1 locus (3p21) in Asian populations. *BMC Genetics,* 7**,** 56.

GIANDUZZO, T. R., HOLMES, E. G., TINGGI, U., SHAHIN, M., MACTAGGART, P. & NICOL, D. (2003) Prostatic and peripheral blood selenium levels after oral supplementation. *Journal of Urology,* 170**,** 870-873.

GILL, J. K., FRANKE, A. A., STEVEN MORRIS, J., COONEY, R. V., WILKENS, L. R., LE MARCHAND, L., GOODMAN, M. T., HENDERSON, B. E. & KOLONEL, L. N. (2009) Association of selenium, tocopherols, carotenoids,

retinol, and 15-isoprostane F(2t) in serum or urine with prostate cancer risk: the multiethnic cohort. *Cancer Causes and Control*.

HATHCOCK, J. (2004) *Vitamin and Mineral Safety,* Washington DC, Council for Responsible Nutrition.

HO, E., COURTEMANCHE, C. & AMES, B. N. (2003) Zinc deficiency induces oxidative DNA damage and increases p53 expression in human lung fibroblasts. *Journal of Nutrition,* 133**,** 2543-2548.

HU, Y., BENYA, R. V., CARROLL, R. E. & DIAMOND, A. M. (2005) Allelic loss of the gene for the GPX1 selenium-containing protein is a common event in cancer. *Journal of Nutrition,* 135**,** 3021S-3024S.

HU, Y. J. & DIAMOND, A. M. (2003) Role of glutathione peroxidase 1 in breast cancer: loss of heterozygosity and allelic differences in the response to selenium. *Cancer Research,* 63**,** 3347-3351.

JABLONSKA, E., GROMADZINSKA, J., SOBALA, W., RESZKA, E. & WASOWICZ, W. (2008) Lung cancer risk associated with selenium status is modified in smoking individuals by Sep15 polymorphism. *European Journal of Nutrition,* 47**,** 47-54.

KIMURA, M., UMEGAKI, K., HIGUCHI, M., THOMAS, P. & FENECH, M. (2004) Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. *Journal of Nutrition,* 134**,** 48-56.

LEIST, M., RAAB, B., MAURER, S., ROSICK, U. & BRIGELIUS-FLOHE, R. (1996) Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. *Free Radical Biology and Medicine,* 21**,** 297-306.

LIPPMAN, S. M., KLEIN, E. A., GOODMAN, P. J., LUCIA, M. S., THOMPSON, I. M., FORD, L. G., PARNES, H. L., MINASIAN, L. M., GAZIANO, J. M., HARTLINE, J. A., PARSONS, J. K., BEARDEN, J. D., 3RD, CRAWFORD, E. D., GOODMAN, G. E., CLAUDIO, J., WINQUIST, E., COOK, E. D., KARP, D. D., WALTHER, P., LIEBER, M. M., KRISTAL, A. R., DARKE, A. K., ARNOLD, K. B., GANZ, P. A., SANTELLA, R. M., ALBANES, D., TAYLOR, P. R., PROBSTFIELD, J. L., JAGPAL, T. J., CROWLEY, J. J., MEYSKENS, F. L., JR., BAKER, L. H. & COLTMAN, C. A., JR. (2009) Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA,* 301**,** 39-51.

LYONS, G., JUDSON, G., STANGOULIS, J., PALMER, L., JONES, J. & GRAHAM, R. (2004) Trends in selenium status of South Australians. *Medical Journal of Australia,* 180**,** 383-386.

MEPLAN, C., CROSLEY, L. K., NICOL, F., BECKETT, G. J., HOWIE, A. F., HILL, K. E., HORGAN, G., MATHERS, J. C., ARTHUR, J. R. & HESKETH, J. E. (2007) Genetic polymorphisms in the human selenoprotein P gene determine the response of selenoprotein markers to selenium supplementation in a gender-specific manner (the SELGEN study). *FASEB Journal,* 21**,** 3063-3074.

NATIONAL HEALTH AND MEDICAL RESEARCH COUNCIL, N. (2005) *Nutrition reference values for Australia and New Zealand,* Canberra, Australian government, Department of Health and Aging.

PETERS, U., FOSTER, C. B., CHATTERJEE, N., SCHATZKIN, A., REDING, D., ANDRIOLE, G. L., CRAWFORD, E. D., STURUP, S., CHANOCK, S. J. & HAYES, R. B. (2007) Serum selenium and risk of prostate cancer-a nested case-control study. *American Journal of Clinical Nutrition,* 85**,** 209-217.

REILLY, C. (1996) *Selenium in food and health,* London & New York, Blackie Academic & Professional.

SCHRAUZER, G. (2000) Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *Journal of Nutrition*, 130**,** 1653-1656.

YANG, G. & ZHOU, R. (1994) Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China. *Journal of Trace Elements and Electrolytes in Health and Disease*, 8**,** 159-165.

Appendix

I. Wu J, Salisbury C, Graham R, Lyons G, Fenech M. The effect of selenium, as selenomethionine, on genome stability and cytotoxicity in human lymphocytes as measured by the cytokinesis-block micronucleus cytome assay. ***Mutagenesis.*** 2009 May; 24(3):225-32.

II. Wu J, Lyons G, Graham R, Fenech M. Increased consumption of wheat biofortified with selenium does not modify biomarkers of cancer risk, oxidative stress or immune function in Australian males. ***Environmental Molecular Mutagenesis.*** 2009 July; 50 (6):489-501

III. CSIRO FFQ

IV. ACCV FFQ

Wu, J., Lyons, G.H., Graham, R.D. and Fenech, M.F. (2009) The effect of selenium, as selenomethionine, on genome stability and cytotoxicity in human lymphocytes measured using the cytokinesis-block micronucleus cytome assay
Mutagenesis, v.24 (3), pp. 225-232, May 2009

Wu, J., Salisbury, C., Lyons, G.H., Graham, R.D. and Fenech, M.F. (2009) Increased consumption of wheat biofortified with selenium does not modify biomarkers of cancer risk, oxidative stress, or immune function in healthy Australian males
Environmental and Molecular Mutagenesis, v.50 (6), pp. 489-501, July 2009

NOTE:
Appendices III- IV are included in the print copy of the thesis held in the University of Adelaide Library.