An analysis of the protective effects of Selenium on porcine jejunal epithelial cells following Cadmium-induced oxidative DNA damage.

A thesis submitted for the Degree of

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by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Acknowledements:

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Abbreviations

AB	Alamar blue
AIF	Apoptosis-inducing factor
ALS	Amyotrophic lateral sclerosis
As	Arsenic
BER ORA	Base Excision Repair oligonucleotide retrieval
BER	Base excision repair
Ca	Calcium
CAT	Catalase
Cd	Cadmium
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
Cu	Copper
CYCS	Cytochrome c
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DOI	Iodothyronine deiodinase
DRMB	DNA repair molecular beacon
DSB	Double strand DNA break
EFSA	European Food Safety Authority

EPIC	European Prospective Investigation into Cancer and Nutrition
FapyG	Formamidopyrimidine
Fe	Iron
FM-HCR	Flow-cytometric host cell reactivation assay
FPN1	Ferroportin 1
GH	5-guanidinohydantoin
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSH-Px	Glutathione peroxidases
GS-Se-SG	Selenodiglutathione
Hg	Mercury
HR	Homologous recombination
HSF1	Heat shock factor 1
HSR	Heat shock response
Ia	Iminoallantoin
IARC	International Agency for Research in Cancer
IDL	Insertion/deletion
LP	Long patch
MAP	MUHYT associated polyposis

MHD	Mulberry heart disease
MM	Mismatch
MMPs	Matrix Metalloproteinases
MMR	Mismatch repair pathway
MPT	Mitochondrial permeability transition
mRNA	Messenger RNA
Mt	Metallothionein
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NGS	Next generation sequencing
NHEJ	Non-homologous end-joining
NMR	Nuclear magnetic resonance
NPC	Nutritional Prevention of Cancer Trial
Pb	Lead
PCB	Polychlorinated biphenyl
PUFA	Polyunsaturated fatty acid
RASFF	Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species

SAM	S-Adenosyl methionine
SBS	Sequencing by synthesis
Se	Selenium
SeCys	Selenocysteine
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SelW	Selenoprotein W
Se-M	Selenomethionine
SeMet	Selenomethionine
Se-Na	Sodium selenite
Se-Ni	Sodium selenite
Se-Y	Selenium yeast
SOD	Superoxide dismutase
SP	Short patch
Sp	Spiroiminodihydantoin
StAR	Steroidogenic acute regulatory
SUMO2	Small Ubiquitin-like Modifier-2
TLS	Trans-lesional synthesis
TRx	Thioredoxin reductase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UBB	Ubiquitin

UPP	Ubiquitin-proteasome pathway
V(D)J	Variable, diversity and joining
WMD	White muscle disease
Zn	Zinc

Abstract

An analysis of the protective effects of Selenium on porcine jejunal epithelial cells following Cadmium-induced oxidative DNA damage.

Sarah Lynch

The increasing global demand for cheap, available protein has resulted in substandard animal feeds which generally lack essential minerals and often contain toxic levels of heavy metals. Cd is a bio-toxic heavy metal and exposure causes DNA strand breakage, mutations and inhibition of DNA synthesis. Selenium (Se) is an essential trace element that possesses antioxidant properties. In this study, the potential ameliorative effects of various Se sources on Cd induced damage were investigated. The impact of several forms of Se supplementation on cell viability and DNA damage in porcine primary jejeunal (IPEC-J2) cells following Cd exposure was analysed. It was observed that Se protective effects were both composition- and dose-dependent, with organic (Se-M) and Se yeast (Se-Y) affording the highest level of protection from Cd-induced oxidized DNA damage while inorganic Se exhibited cytotoxic effects. The potential mechanism(s) of action of Se-Y sources on Cd-induced damage were then compared. Thus, the antioxidant activity, the extent of apoptosis-associated DNA fragmentation, the DNA repair capacity and the transcriptome of IPEC-J2 cells were analysed following pre-incubation with each of three different commercial Se-Y preparations. The data obtained confirmed the ability of different Se-Y preparations to enhance a range of cellular mechanisms that protected IPEC-J2 cells and clearly illustrated the difference in bioefficiency of different Se-Y sources. Overall, the results presented are relevant to the Agri-Food industry, illustrating the negative impact of traditional inorganic Se supplementation and highlighting the significant benefits to using Se-Y in animal feed products for the protection of intestinal cells against Cd induced oxidative DNA damage.

1) Introduction

The value of global agricultural outputs contributed by livestock production totalled 40% in 2016, with the livestock industry creating employment and ensuring food security for almost 1.3 billion people. The USA is the leading meat producing country, followed by Brazil with the European Union being the third largest meat producer in 2019. Worldwide, approximately 65 billion chickens, 1.5 billion pigs, and 330 million cattle and buffaloes are reared annually for meat production (1). The magnitude of global animal production is directly connected to human dietary requirements which have significantly increased in recent times. The global need for animal production has resulted from an increased consumption of animal products as expendable incomes escalate (2). The agri-food division is Ireland's most important indigenous industry and plays a crucial role in the growth and stability of that country's economy. The sector generated a turnover of €26 billion thereby providing 7.8% of gross national income (GNI) in 2017. The products of the Irish agri-food industry are exported to over 180 countries globally with exports equalling €13.6 billion in 2017 alone (3). Dairy, beef and pig meat are the top three Irish exports with the UK being the main destination for Irish agri-food exports accounting for 37% of exports followed by 32% to EU markets; and 31% to international markets (4). In 2017, an estimated 537 thousand tonnes of beef worth approximately €1.85 billion was exported from Ireland and a further 189,000 cattle were exported live from Ireland worth an estimated €100 million (5). Pig production in Ireland ranks third in terms of revenue generation preceded by the beef and dairy sectors and in 2017 accounted for 8% of Gross Agricultural Output. In the same year, pig meat worth an estimated €792 million was exported from Ireland to EU and international markets. As part of the Food Wise 2025 initiative that aims to strengthen the Irish agricultural economy, the Irish pig meat sector is aiming for substantial growth (3). One of the main reasons for the sustainable increase in pig farming is the significant improvement in feeding and genetics. Animal feedstuffs are frequently

contaminated with environmental contaminants including pesticides, industrial pollutants, radionuclides and heavy metals. In 1999, high levels of dioxins were detected in meat products and eggs from several farms after dioxin-contaminated animal fat was added to animal feed. The pig meat industry took a significant hit in 2008 when pork products had to be recalled from the market after dangerous levels of dioxins were discovered and farmers suffered a loss of approximately €20 per pig (6). Such incidences of animal feed contamination therefore have a direct impact on the agri-food industry and the Irish agricultural economy.

1.1) Selenium Biochemistry

Selenium (Se) is a metalloid belonging to group 16 (VIA) of the periodic table, it is a member of the chalcogen family and can exist in four oxidation states in nature: selenide (Se(-II)), elemental Se (Se(0), selenite (Se(IV)), and selenate (Se(VI)) (7). Se is an essential trace element in animal health, which plays a critical role in antioxidant defence system, immune response and thyroid function. Selenocysteine (SeCys) is the principal form of Se found in the body, it is the 21st proteinogenic amino acid which functions as the building block of selenoproteins. SeCys is found in the active centre of selenoproteins, however in selenomethionine (Se-M), SeCys is incorporated unspecifically into general proteins and methylated Se-compounds (8). The incorporation of SeCys in the formation of a selenoprotein is controlled by the UGA codon in mRNA under specific conditions (9). Co-translational incorporation of SeCys into selenoproteins requires special *trans*-acting protein factors, Sec-tRNA$^{[Ser]Sec}$ and a *cis*-acting SeCys insertion sequence (SECIS) element (10) (see Fig.1.1)

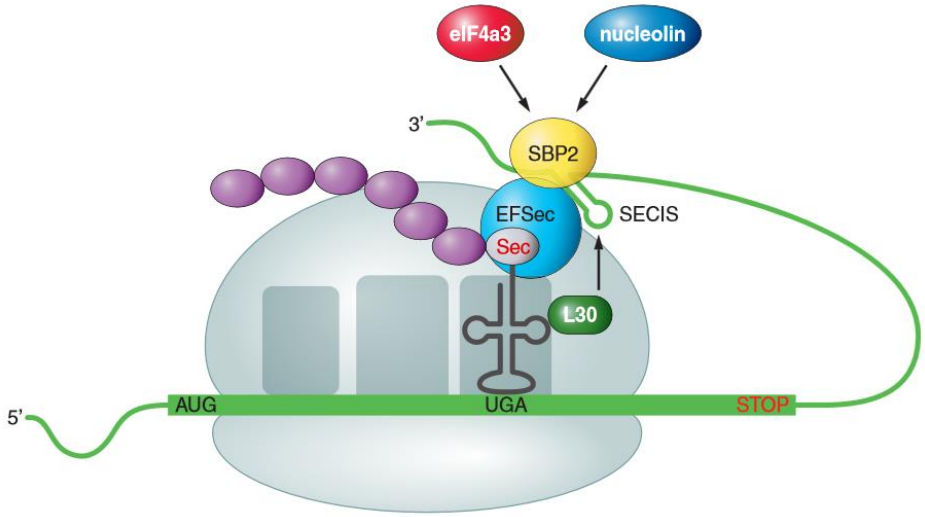

Figure 1.1: Mechanism of SeCys incorporation in eukaryotes. When a ribosome encounters the UGA codon, SeCys machinery interacts with the canonical translation machinery and prevents premature termination. SECIS elements serve as the factors that dictate recoding of UGA as SeCys. Sec-tRNA translates UGA as SeCys. SECIS binding protein 2 (SBP2) interacts with Sec-specific translation elongation factor (eEFSec), which recruits SeCys-tRNA and facilitates incorporation of SeCys into the growing polypeptide. Ribosomal protein L30 has been predicted to constitute a part of the basal SeCys insertion machinery, while nucleolin and eukaryotic initiation factor (eIF4a3) serve as regulatory proteins that modulate synthesis of selenoproteins and may contribute to the hierarchy of selenoprotein expression (10).

The physiological functions of Se are thought to be mediated through selenoproteins and Se status is understood to regulate selenoprotein expression (11). Over 30 SeCys containing selenoproteins have been described (9), selenoproteins can be classified into two main categories based on the location of the SeCys in the selenoprotein polypeptide. The selenoenzyme Glutathione peroxidase (GPx) is a member of group one, where the SeCys is present on the N terminal of the function domain, whereas the selenocysteine in group two is located on the C-terminal as is the case with thioredoxin reductase (TRx) (12). Selenoproteins have a major effect on a wide range of biological functions such as reproduction, production of thyroid hormones and brain function as outlined in Table 1.1.

Table 1.1: Function of various selenoproteins, adapted from (13).

Selenoprotein	**Biological Function**
Glutathione Peroxidase	GPx
GPx1/GPx2/GPx3/GPx6	Glutathione dependent detoxification of hydrogen peroxide
GPx4	Inhibition of lipid peroxidation
Iodothyronine deiodinase	DOI
Type I Iodothyronine deiodinase (DOI1) **Type II Iodothyronine deiodinase (DOI2)** **Type III Iodothyronine deiodinase (DOI3)**	Regulation of thyroid hormone activity by reductive deiodination
Thioredoxin Reductase	
TRx1	Reduction of the oxidized form of cytosolic thioredoxin
TRx2	Formation/Isomerization of disulphide bonds during sperm maturation
TRx3	Reduction of mitochondrial thioredoxin and glutaredoxin 2
15 kDa selenoprotein (Sep 15)	Quality of protein folding
Selenoprotein H	Regulation of GSH synthesis and phase II detoxification enzymes
Selenoprotein K	ER-associated degradation of misfolded proteins
Selenoprotein M	Rearrangement of disulfide bonds in the ER-localized proteins
Selenoprotein N	Regulation of intracellular calcium mobilization
Selenoprotein P	Se transport to peripheral tissues and antioxidant function Repair
Selenoprotein R	Repair of oxidized methionines in proteins ER-associated
Selenoprotein S	ER-associated degradation of misfolded proteins
Selenophosphate synthetase	Synthesis of selenophosphate
Selenoprotein W	Redox regulation of 14-3-3 protein

1.1.1 Se deficiency and associated disorders:

Many studies have confirmed that Se exhibits a U shaped dose response (14–16), meaning that either excessive or insufficient quantities of the trace element leads to an increased risk of mortality. According to EFSA regulations the optimal concentration of Se yeast (SeY) for supplementation of animal feed is between 0.2-0.4 ppm Se (17, 18). Concentrations lower or greater than this optimal range can lead to adverse effects in ruminants. Therefore, Se deficiency can have a severe effect on animal health and mortality. White muscle disease (WMD) is a common consequence of Se deficiency especially in young, rapidly developing animals. WMD causes degenerative alterations in skeletal muscles fibres resulting in abnormal movement and postural positions; it can also affect cardiac muscle fibres leading to sudden death. This degeneration of muscle fibres has been linked to insufficient functioning of the antioxidant system evident by low intramuscular GPx activity, which in turn leads to oxidative damage to skeletal muscle (19). Evidence has shown that gene expression patterns of certain selenoprotein-coding genes in the endoplasmic reticulum correlated with increased apoptosis and elevated oxidative stress in muscles of Se deficient chicks. Results demonstrated a significant down regulation of *sepn1, selt, selk* and *sels* in Se deficient chicks along with an upregulation of proapoptosis-associated gene (*bax, caspase3* and *bcl2*) (11).

Transcription of pro-inflammatory enzymes iNOS and COX-2 and cytokines TNF-α and IL-6 were shown to be upregulated in Se deficient chicken kidneys, proteins that are linked to kidney pathophysiology and abnormal renal function. Increased expression of inflammatory factors was accompanied by elevated serum levels of uric acid and creatine, indications of excretion dysfunction of the kidneys of such Se deficient chickens (20). Insufficient dietary Se levels were shown to downregulate *dio1*, *sepn1*, *sepp1*, *selT*, *sep15* and *txnrd3* mRNA levels in the rat liver and *Gpx3* mRNA levels were reduced to 40% of Se-adequate levels in rat?the kidneys (20, 21). Elsewhere, acute effects on liver hydrogen peroxide metabolism were observed in Se

deficient chickens (22). In that study, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) significantly increased in Se deficient groups indicating liver cell damage and reduced SOD (superoxide dismutase) and CAT (catalase) levels rendered chickens more susceptible to the damaging effects of oxidative stress (have I got that right??). Alterations in expression of Matrix Metalloproteinases (MMPs) have been linked to Se deficiency in the rat model (22). MMPs are the main enzymes responsible for extracellular matrix degradation, which is essential for normal tissue repair. Se deficiency caused decreased expression of TIMP1/3, which play a crucial role in apoptosis inhibition and liver homeostasis and a significant increase in MMP1/3 suggesting liver tissue damage and tumour initiation (22, 23). Se deficiency resulting in decreased antioxidant capacity has been shown to increase the incidence of Mulberry Heart Disease (MHD) in pigs. Without the protective effects of Se against oxidative stress, free radical attack on cellular membranes and elevated calcium influx resulted in mitochondrial calcium overload and muscle fibre deterioration (24).

Sufficient dietary Se intake is essential in the synthesis and metabolism of thyroid hormones and to prevent thyroid gland damage caused by excessive iodide exposure. Se deficiency altered the activity of thyroidal triiodothyronine (T3), thyroidal thyroxine (T4), free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (TSH) in the plasma and the deiodinase (DIO) family in tissues (25). Se deficiency in chickens inhibited the conversion of T4 to T3 and lead to accumulation of T4 and FT4. A significant reduction in deiodinase, glutathione and thioredoxin enzymes was observed in Se deficient chickens (25). Thyroid hormones are important regulators of the reproductive system and alterations of serum levels of thyroid hormones have profound effects on animal reproduction (26). Dietary Se deficiency negatively affected reproductive function in male chickens, indicated by a significant increase in thyroidal thyroxine (T4), free thyroxine (FT4) and thyroid-stimulating hormone (TSH). Fluctuations in thyroid hormones of Se deficient male chickens was

accompanied by an increase in apoptotic cells, and mRNA level of *bax*, *caspase3* and *p53* in the testes (27).

Mastitis is an inflammatory reaction in the mammary gland caused by a bacterial infection and reduces milk yield and quality. Mastitis can reduce reproductive performance, increase the risk of premature culling, and heighten the chance of reoccurrence and herd outbreak, therefore this infection poses a substantial problem for the dairy industry with major economic losses due to production losses and control expenditures (28, 29). Many studies have associated increased incidence of mastitis with Se deficiency in dairy cows (30–32), research also confirmed a connection between exposure to oxidative stress (ROS) and increased incidence of mastitis (33). High energy and oxygen demand during lactation leads to escalated production of ROS, Se deficiency intensifies the imbalance between pro-oxidants and antioxidant defence availability (32). Se deficiency accelerates the production of proinflammatory factors and reduces IL-10 expression which intensifies the inflammatory reaction in mastitis (34). Histopathological changes observed in *S. aureus* induced mastitis, indicated that Se deficiency led to an increase in the number of inflammatory lesions but did not actively induce lesions in the mammary gland, similar results were observed in Lipopolysaccharide-induced mastitis (31, 35).

1.1.2 Se excess and associated disorders

Se plays a crucial role in many biological functions including DNA synthesis, antioxidant defence and immune function. However, there is a narrow margin between Se deficiency and toxicity. Studies have shown that the effects of Se are both concentration and speciation dependent (36). In Europe the recommended daily Se intake for adults is 30µg with EFSA setting the tolerable upper intake at 300µg/day. In humans, Se toxicity results in selenosis

characterised by brittle hair and nails, vomiting and pulmonary oedema. It is difficult to determine the concentration at which Se becomes toxic, as Se toxicity is affected by many factors including the form of Se ingested, the individual's genotype and other dietary components present. Excess Se intake has been associated with the development of chronic degenerative conditions including cardiovascular disease and neurological disorders such as amyotrophic lateral sclerosis (ALS), a fast progressing motor neurone disease. Inorganic Se is reportedly 40 times more neurotoxic than organic Se species and has been hypothesized as a risk factor for the development of ALS (37). Higher levels of inorganic selenite in cerebrospinal fluid and increased albumin bound Se in human serum has been linked with increased risk of development of ALS (38). Neurological disorders associated with excessive Se exposure have also been observed in animals. Progressive peracute forms of Se toxicosis have been widely reported in pig farming (39–41). Muscular tremors, paresis of the hind legs and front limbs, ataxia and anorexia have been observed in affected pigs. In a recent study investigating Se toxicosis caused by addition of commercially available selenite to pig feeds at a concentration of between 1.23 – 2.18 Se/Kg body weight, pig livers were found to be moderately atrophied, with evidence of lung edema in some pigs. Brainstem and mesencephalon changes were observed which resulted in lesions with extensive areas of liquefaction necrosis and it was concluded that the most probable mechanism leading to CNS lesion formation?? was astrocyte edema (40). Recent research has determined an association between high serum Se levels and increased prevalence of diabetes. A recent study revealed a direct relationship between high Se exposure and increased risk of diabetes, this positive correlation between excessive Se and diabetes was found to be independent of both obesity and insulin resistance (42). The link between Se and the development of diabetes has also been shown in animal models; exposure to supranutritional levels of Se in the mouse model led to overproduction of GPx1 which resulted in chronic hyperinsulinaemia by dysregulating islet insulin production and secretion

(43). Se toxicity is also influenced by concomitant presence of other compounds which can have antagonistic or synergistic effects on the target organism (7, 37). The presence of high concentrations of Se can increase arsenic (As) toxicity as the two have similar metabolic conversion pathways and both require GSH and S-Adenosyl methionine (SAM) (44). When present in high concentrations Se and As will compete for limited SAM, which will increase the toxic effects of As as SAM serves as a methyl donor for the detoxification of both compounds (44, 45). This effect has also been proven *in vivo* using a rat model, where Se^{IV} compromised the monomethylation of As (46).

1.2 Se and Cancer

It has been reported that Se may exhibit anticarcinogenic activities, this has been supported by three types of evidence. Firstly, epidemiologic studies and large scale clinical trials have detected an inverse relationship between nutritional Se status and cancer risk, suggesting that inadequate Se status may increase cancer risk (9, 47) Supplementation with supranutritional levels of Se has been shown to reduce tumorigenesis in various cell and animal models (48–51). Many epidemiological studies and clinical trials have focused on investigating the chemopreventive potential of Se compounds (see Table 1.2).

Table 1.2: Recent epidemiological and clinical trial studies focused on elucidating the relationship between Se and cancer incidence.

Study name	Population	Type of cancer	Outcomes
The National Health and Nutrition Examination Survey (NHANES III) (52).	13,887 adult participants served as a representative sample of the US population.	Cancer (prostate, lung, and colorectal cancer) and cancer mortality.	Increasing Se levels were associated with a decrease in cancer associated deaths up to 130 ng/mL.
Se is inversely associated with bladder cancer risk: a report from the Belgian case–control study on bladder cancer (53).	178 cancer cases and 362 control participants.	Bladder cancer.	An inverse relationship between Se levels and bladder cancer incidence was observed. Patients with serum Se concentrations lower than 82 µg/L had a greater risk of bladder cancer.
Levels of Se, zinc, copper, and antioxidant enzyme activity in patients with leukemia (54).	49 participants with various types of leukemia.	Leukemia.	Low Se concentrations and both elevated GPx activity and copper levels were detected in leukemic patients.
The Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) (55).	12,741 French adults. 7,713 females aged 35–60 years; 5,028 males aged 45–60 years).	All cancers (basal cell carcinoma was excluded).	A decrease in the risk of cancer in the Se supplemented group was seen only in men with serum values above the cut-off limit concentrations.

The Nutritional Prevention of Cancer Trial (NPC) was the first double-blind, placebo-controlled, randomized trial to investigate the chemopreventive effects of Se supplementation on cancer risk in the western world. The trial was designed to investigate the potential of Se-Y (200 µg/day) to prevent the reoccurrence of nonmelanoma skin cancer (56). The primary outcomes were the incidences of basal cell and squamous cell skin cancer, while the secondary

endpoints included all-cause mortality and total cancer mortality, total cancer incidence, and the incidences of lung, prostate, and colorectal cancers (47, 57). Throughout the blinded period of the trial it was continuously demonstrated that Se treatment did not protect against development of basal or squamous cell carcinomas, in fact there was a significantly increased risk of squamous cell carcinomas and a slightly increased risk of basal cell carcinomas following Se treatment (47, 58). In contrast, data gathered concerning the secondary endpoints of the trial revealed statistically significant decreases in the incidence of prostate cancer (52%), lung cancer (26%), colorectal cancer (54%), total cancer incidence (25%) and total cancer mortality (41%) (56, 58). Observation of the secondary outcomes of the NPC trial alongside the secondary results of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) which demonstrated a 32% decrease in prostate cancer risk following vitamin E supplementation led to the design of *The Se and Vitamin E Cancer Prevention Trial* (SELECT) (15, 47).

SELECT was a phase III, large scale, double blind, randomized clinical trial that investigated the chemopreventive effect of Se and vitamin E on prostate cancer in 35,335 American men (59). The results revealed that administration of 200µg Se/day in the form of Se-M did not reduce the risk of prostate cancer while dietary supplementation with vitamin E significantly increased the incidence of prostate cancer (59, 60). There were no significant differences in any predetermined secondary endpoints which included cardiac events and diabetes. The results from the SELECT trial were inconsistent with the results observed in the NPC trial, one possibility for the conflicting results between the two trials is the underlying difference in the chosen populations. The NPC trial recruited only patients with nonmelanoma skin cancer whereas the SELECT trial more accurately reflected the general population (47). Another confounding factor that may have influenced the contrasting results between the two studies was the difference in Se exposure levels of the trial subjects. The NPC trial included

participants with a broad Se exposure range (<106 µg/L - >123.2 µg/L), whereas the SELECT trial included almost no participants from the lower Se exposure range. The SELECT trial administered Se in the form of Se-M whereas the NPC trial supplemented participants diets with Se-Y (9). The results from the European Prospective Investigation into Cancer and Nutrition (EPIC) also found no association between Se levels and cancer risk. The EPIC study indicated that low plasma Se levels are not associated with increased prostate cancer risk. However, the range of Se concentrations may have been too narrow to detect a significant association (61).

Se can also act as a prooxidant, inducing apoptosis through the generation of oxidative stress and has attracted interest as a potential anticancer agent. Se has been shown to act as a redox modulator with a high tumour specificity (62). Various Se metabolites are produced after metabolism of dietary Se compounds by several distinct pathways which determine their biological activity. The Se metabolites that are critical in redox cycling and generate hydrogen peroxide and superoxides resulting in ROS production are the prooxidants hydrogen selenide (HSe^-) and methyl selenol (CH_3Se^-). CH_3Se^- is also produced when redox inactive Se metabolites such as methylselenocysteine are cleaved by β lyase further increasing the oxidative environment and inducing apoptosis and DNA fragmentation in cancer cells (62, 63). Studies have shown that certain Se compounds and metabolites can inhibit angiogenesis and cut off blood supply to tumours. Methylseleninic acid (MSA) inhibited angiogenesis at nutritional concentrations by downregulating integrin β3 at mRNA and protein levels in human umbilical vein endothelial cells. Inhibition of phosphorylation of AKT, IκBα, and NFκB was observed suggesting that MSA exposure also disrupts the clustering pattern of integrin β3 (64). This angiogenic effect was also detected in diethylnitrosamine-induced hepatocarcinoma in rats that were exposed to Se enriched malt by the downregulation of vascular endothelial

growth factor (VEGF) in the tumor tissue. Rats treated with inorganic selenite did not show reduced VEGF expression (65).

1.3 Se forms available for nutritional supplementation

Se is primarily found in plant sources and is thereby transferred to the food chain upon consumption. Therefore, the Se content of the soil and its availability is a highly important factor for Se intake. However, Se content of soils and its availability to plants is highly variable across the world (66). Since the discovery of the connection between Se and animal health, there have been many studies carried out investigating the potential beneficial effects of Se supplementation. In European countries, animal feed is frequently supplemented with either inorganic Se (sodium selenite, sodium selenate) or organic Se sources (Se-M or Se yeast (Se-Y)) due to the low availability of Se in some soils. Animal feedstuffs are most commonly supplemented with inorganic Se as it is less costly than organic Se sources. During digestion of inorganic Se, complexes may be formed with free mineral ions released during the process rendering the Se difficult to absorb (67, 68).

The duodenum is the main site of absorption for the majority of ingested Se, followed by the jejunum and the ileum (67). Organic and inorganic forms of Se are not metabolized alike. The inorganic Se forms sodium selenite and sodium selenate are passively absorbed in the small intestine by simple diffusion along the concentration gradient, while organic sources are actively absorbed by amino acid transport mechanisms (8, 67). Upon absorption of sodium selenite, selenite is immediately non-enzymatically reduced via formation of selenodiglutathione (GS-Se-SG) to selenide. Sodium selenate is not immediately reduced to selenide as it requires several passes through the liver to be metabolised to selenide and a considerable portion of absorbed selenate is excreted directly and unchanged in urine before

being reduced. However, Se-M is converted into SeCys and can be incorporated non-specifically into proteins, as it freely substitutes for methionine in protein synthesis. Se-M can also be converted into selenide and can partake in the first metabolic pathway which is responsible for selenoprotein production which includes the co-translational biosynthesis of SeCys and its incorporation into specific selenoproteins (8, 67, 69). The second pathway is that Se in excess of these needs enters an excretory pathway, and methylation and sugar-derivation of selenides form the major excretory products (69).

Unlike the organic forms of Se, in which Se is in the reduced state (selenide: Se^{2+}), the inorganic salts contain Se in oxidized forms (selenite: Se^{4+}; selenate: Se^{6+}). Upon absorption, the higher-valence forms are reduced to the selenide state using reducing equivalents from reduced glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH). In contrast, the organic forms (Se-M, SeCys) release Se in the selenide state as a result of catabolism. The Se from Se-M, consumed in the form of food proteins and/or dietary supplements, is thus transferred to form SeCys. Studies have shown that the absorption of Se is significantly lower in ruminants than in monogastric animals. Retention of sodium selenite was found to be 77% in swine and only 29% in sheep, research suggesting that inorganic Se is reduced to insoluble selenide or elemental Se by rumen microorganisms leading to excretion of Se thus decreasing absorption and retention levels (70). Se retention in lambs fed either a diet containing sodium selenite or Se yeast demonstrated that feed supplementation with Se from Se-yeast resulted in higher absorption of Se from the digestive tract and greater Se retention than when sodium selenite was used as supplement (8). Inorganic Se is greatly reduced in the rumen and becomes less available for absorption than organic Se. Permeability studies in Caco-2 cells illustrated Se-M and SeCys showed a larger absorptive flux than exsorptive flux, the organic Se sources demonstrated twice the absorptive flux of inorganic selenate (71). Research confirms that inorganic Se in the form of sodium selenite results in a

strong genotoxic effect in HepG2 liver cells after oxidative challenge by lead (Pb), while supplementation with Se-Y and subsequent exposure to Pb resulted in a significant genoprotective effect (72). Other literature agrees that Se-Ni is highly cytotoxic to cells and causes DNA strand breaks, cell membrane leakage and cell detachment (73).

Differences in the reproductive performance of hens fed Se-Y or inorganic Se have been reported. Evidence shows that in female reproductive tissues Se-Y supplementation but not sodium selenite supplementation, enhances energy production and protein translation, while genes associated with energy production and protein translation were down regulated in hens receiving sodium selenite treatment. Genes encoding several subunits of the mitochondrial respiratory complexes, ubiquinone production and ribosomal subunits were upregulated during Se-Y supplementation whereas, a decrease in respiratory complex related gene transcripts were observed in sodium selenite treated hens (74). Another study conducted on beef heifers also illustrated the differences in gene expression in relation to Se source in feed. Upregulation of mitochondrial gene expression was noted in both organic Se and inorganic Se treated beef heifers, however, the expression of a protein with antiviral capacity was downregulated in inorganic Se treated animals. Decreased mRNA levels from genes encoding proteins upregulated during oxidative stress were observed in the organic Se supplemented group, indicating a protective effect of organic Se on oxidative stress (75). Organic Se treatment of mice in the form of Se-Y significantly reduced the expression of Gadd45b in tissues and also reduced GADD45B protein levels in liver, in comparison to inorganic Se. Se-M supplementation of weaning pigs has been shown to increase growth performance, plasma Se and antioxidant ability (76). Organic Se supplementation had an ameliorative effect on heavy metal induced cytotoxicity and oxidative DNA damage in a porcine gut intestinal model while inorganic Se in fact enhanced the negative effects of heavy metal induced damage (36).

1.3.1) Se Yeast:

In recent years, due to the increasing evidence of the harmful effects and low bioavailability of inorganic Se there has been increased interest in the use of Se enriched yeast (Se-Y) preparations in animal feed products. Se-Y is an appealing Se source for nutritional supplementation due to its activity in selenoprotein synthesis and its low toxicity. In addition, there is evidence that Se-Y is highly bioavailable, bioactive and has the ability to increase selenoenzyme activity (77). Recent studies have provided evidence that supplementation with organic Se affords protection against oxidative DNA damage, increases immune response and positively effects cell proliferation and viability (30, 72, 78–80).

Se can be bio-transformed by yeast cells into Se-M by incorporation into proteins non-specifically by replacing sulfur in methionine, thus producing Se-M (81). However, the mechanism of accumulation of Se and its conversion into cellular structures is not fully understood. The ability of the yeast cells to absorb Se increases under conditions of sulfur deficiency. In the first stage of Se metabolism in yeast cells, sodium selenite is reduced to hydrogen selenide, this reaction is catalysed by sulphate reductase with NADPH acting as the reducing agent. Hydrogen selenide (H_2Se) has the ability to penetrate into the cell via a passive way and is the major intermediate metabolite involved in the synthesis pathway of all forms of Se produced in microbial cells. The first step in the conversion of H_2Se into organic Se compounds is the biosynthesis of selenohomocysteine. In the following steps, selenohomocysteine is subjected to a methylation process, resulting in the formation of Se-M (82). As well as amassing high Se concentrations, yeast cells utilize sugars and organic acids to produce biomass with high protein levels (83). Studies examining the Se binding efficiency of different yeast strains have focused mainly on the Se tolerant Saccharomyces cerevisiae and *Candida utilis* (also known as Torula yeast) (84, 85). To date, only Se tolerant strains of *S. cerevisiae* and *C. utilis* have been used by commercial manufacturers for Se-Y production as

they have been granted GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (FDA) (83) and are approved for animal and human use by the European Food Safety Authority (EFSA; EFSA regulations specify that the total Se concentration in animal feed must not exceed 0.4 ppm and that supplementation with exogenous Se must not exceed 0.2 ppm) (17, 86). Animal feed premixes, which include all the minerals and vitamins essential in the animal diet, are manufactured by premix companies who formulate these mixtures and they label their products a with recommended levels per ton of feed. These premixes are then purchased by feed companies for blending into commercial rations which are then sold to farmers to feed as they wish or a farmer who uses their own grain can formulate their own rations and be assured their animals are getting the recommended levels of minerals and vitamins (17, 87).

The manufacturing process for the production of Se-Y in order to yield a Se-Y product that is suitable for mammalian consumption generally follows the subsequent set of steps 1) cultivation of a food grade yeast under aerobic growth conditions, 2) incremental supplementation with a carbon nutrient source, 3) addition of a water soluble Se source, 4) recovery of the Se-Y once it has reached a predetermined level, 5) removal of extracellular nutrients and Se salts from the Se-Y product by a series of wash steps, 6) pasteurisation and drying of the yeast cells to recover a dried Se-Y product (see Fig. 1.2).

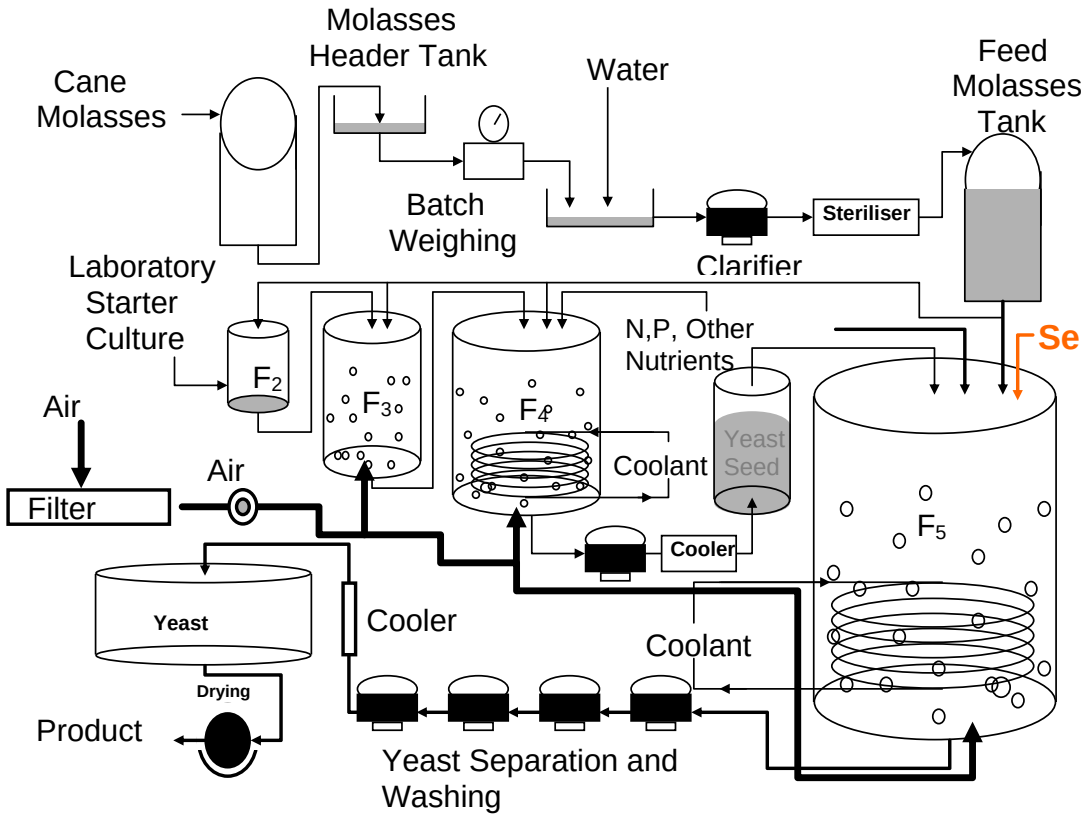

Fig. 1.2: Manufacturing processing steps for the production of a dried Se-Y preparation that is safe for mammalian consumption, adapted from (88).

Although the main Se component of Se-Y is Se-M, it has been observed that gene profiles differ in response to Se-Y and Se-M supplementation in a mouse model, suggesting that Se-Y comprises several different protein-bound Se compounds in addition to Se-M (89). It has also been reported that the form of Se-M which is used as an additive in animal feed is a synthetic form of L-Se-M. The latter contains D-Se-M as an impurity which is not metabolised efficiently and can build up in organs and tissues leading to toxic effects in the body (90). A study in which the digestion and oxidation of different Se compounds was analysed, concluded that Se-M concentrations decreased in the small intestine coupled with the appearance of the oxidation product Se-MetO (91). Se-rich yeast can be characterized by the Se-metabolic profile (selenometabolome), which is characteristic of yeast strain and fermentation parameters. Just

as each yeast strain has its own unique genetic characteristics so do the Se-Y products produced from different yeast strains (92). The selenometabolome is classified as the fraction of water soluble, low molecular weight Se-metabolites that occur in Se-Y. These Se-metabolites comprised 10-20% of available Se in Se-Y. To date, various speciation studies have identified over 60 low molecular weight selenized species from different Se-Y suppliers (93). Each Se-Y product has its own unique selenometabolome therefore, the selenometabolome has become a precious fingerprint of the origin of the preparations available on the market and of the reproducibility of the production process (94). The distinctive nature of the selenometabolome of each individual Se-Y product may suggest that a particular therapeutic activity or toxicity may be linked to a specific species on the selenometabolome (95).

Se-Y supplementation has been shown to improve some morphometric characteristics of villi in duodenum and jejunum in ruminants. Se supplementation was shown to increase cell proliferation in epithelial tissue of young goats and beef steers, increased villi size has been linked to increased GPx levels (96, 97). Swine fed high dietary Se levels as Se-Y accumulated greater Se in plasma and tissues compared to those fed the same levels of selenite. In the selenite supplemented group, toxicity ensued more rapidly and symptoms were more severe than the Se-Y supplemented group (98). Increased milk yield and milk Se levels were observed in dairy cows fed a Se-Y supplemented diet. Rumen fermentation was positively affected and improved digestibility of feed nutrients, suggesting that Se-Y stimulated digestive microorganisms or enzymes (99). This agrees with another experiment conducted on thirty commercial herds across the US, dairy cows were fed Se-Y supplemented diets which increased productivity and protein efficiency (100). Se-Y supplementation in lambs increased digestibility of dry matter, organic matter, crude protein, neutral detergent fiber, acid detergent fiber and nutrient digestibility increased (101). Improved birthrate and increased litter weight of piglets was observed following Se supplementation as well as significant improvement of

liver function (102).. In the mouse model Se-Y supplementation reduced expression of GADD45b and also decreased GADD45b protein levels in the liver, while sodium selenite and Se-M had no effect GADD45b expression levels, this suggests that only Se-Y supplementation was associated with DNA damage protection (89).

1.4.1) Selenium role in antioxidant defense system:

Aerobic organisms possess a complex antioxidant defense system to neutralize reactive oxygen species (ROS), however inadequate functioning of this antioxidant defense system results in inefficient removal of ROS and can lead to severe damage to biological macromolecules, tissue injury and metabolic malfunction (103). Increased levels of ROS can lead to oxidative damage to DNA, in the form of alkali-labile sites, formation of modified DNA bases and sugar moieties single and double strand breaks, and protein-DNA crosslinks (104). It has been reported that Se status directly effects the functioning of many antioxidant enzymes including GPx, TRx, iodothyronine deiodinases (DIO) and catalase (CAT). Glutathione (GSH) is a ubiquitous cellular tripeptide with a sulfhydryl group which neutralises ROS and acts as a cofactor for many Se dependent antioxidant enzymes, such as GPx and glutathione-S-transferase (GST) (105).

The antioxidant properties of Se and its role in the antioxidant defense system have been investigated in animal nutrition for prevention and treatment of ROS-induced pathologies. There is evidence that several of these selenoproteins including GPxs, selenoproteins P, W, and R, and thioredoxins have antioxidant activities (106). Se compounds exhibit both prooxidant and antioxidant behavior, regulation of the redox state is essential for cell proliferation, DNA synthesis and repair, cell viability and organ function (107). GPx1–3 are involved in the reduction of hydrogen peroxide and organic hydroperoxides, while GPx4

directly reduces phospholipids and cholesterol hydroperoxides (108, 109). GPx4 suppresses lipid peroxidation and apoptosis during embryogenesis, it also plays a vital role in sperm maturation and is an important component in the sperm's helical mitochondrial capsule which is essential for motility (108, 110). In ruminants Se-yeast supplemented diet significantly affected rumen fluid enzyme activity. Se level and GPx activity was shown to be significantly higher and malondialdehyde (MDA) concentration was significantly lower in bacterial and protozoal fractions of the rumen (68) . Severe Se deficiency caused almost total loss of GPx1 activity and mRNA in the rat liver and heart, while GPx4 activity was reduced by 75 % in the liver and 60 % in the heart in the mouse model (111).

Like GPx, TRx is a oxidoreductase belonging to the pyridine nucleotide disulfide oxidoreductase GSSG reductase (GR) protein superfamily of dimeric flavoenzymes (112). Three mammalian TRx selenoproteins have been identified, these include TRx1 which functions in the cytosol and nucleus and has a large number of functions in cellular redox control and antioxidant defense such as providing reducing power to RNR (ribonuclease reductase) which is crucial for DNA synthesis. Under conditions of inflammation and oxidative stress TRx1 is secreted from cells and can be detected in plasma (113). Thioredoxin interacting protein (TXNIP) is an endogenous molecule that negatively regulates TRx. In response to oxidative stress TXNIP moves from the nucleus into the cytosol or mitochondria, reduces the binding of Trx1/Trx2 with ASK1 and resulting in a ASK1-mediated signaling pathway plays a vital role in stress responses, including cell death, differentiation, and production of inflammatory cytokines (108, 114).

TrxR2 functions in the mitochondria and is critical in controlling ROS in unison with the GSH system. Mice with cardiac specific deletion of Trx2 produced spontaneous dilated cardiomyopathy resulting in mortality due to heart failure at young age (115). Evidence has shown that TrxR2/Trx2/Trx3 system is crucial in the regulation of H_2O_2 flux from heart

mitochondria under the physiological mode of respiration. The functioning of the TRx system is essential for maintaining the redox environment and therefore overall cell viability and mitochondrial function (116). TrxR3 (also called Thioredoxin glutathione reductase, TGR) functions in testis and is a TRx isozyme that has only been detected in mouse testes (10, 117). The deiodinases (DIO) are peroxidase enzymes involved in synthesis, metabolism, activation or inactivation of thyroid hormones (118). DIO1 and DIO2 which catalyze the removal of an iodine residue from the pro-hormone thyroxine (T4) molecule, producing either the active form triiodothyronine (T3; activation) or inactive metabolites (reverse T3; inactivation) (119). Type 3 iodothyronine selenodeiodinases localized in the plasma membrane are responsible for the inactivation of T4 and T3 which occurs via deiodination of the inner ring (118). Se deficiency in animals can result in significant reduction in T3, an increase in T4 and a reduction in the activity of the liver 5'DI type I (120). Se supplementation in lambs resulted in an elevated blood GPx activity and serum T3 levels and a reduction in serum T4 amount compared to non-supplemented control lambs (121).

1.4.2) Immune Function

Organic Se supplementation has been shown to induce immunostimulant effects including; an increase in proliferation of activated T cells, heightened cytotoxic lymphocyte-mediated tumour cytotoxicity and natural killer cell activity (9). One study employed Se-M treatment to evaluate the effect of organic Se supplementation on Porcine Circovirus type II (PCV2) replication in PK-15 porcine kidney cells. The findings of this study demonstrated that DL-Se-M inhibited PCV2 replication in PK-15 cells in a concentration dependent manner within the range of 4-16µmol/L. A significant increase in GPx levels was observed in this study as a result of Se-M supplementation. This increase in GPx levels may suggest that the inhibitive effect of

Se-M on PCV2 replication is mediated through enhanced antioxidative activity of GPx (122). A study investigating the effect of organic Se supplementation on Taura Syndrome Virus (TSV) resistance found similar results to the above mentioned study, which further corroborated the role of GPx activity in Se mediated viral resistance (123). Se supplementation of footrot affected sheep did not prevent the disease but Se treatment did restore innate and humoral immune functions. Se-Y treatment has been previously shown to improve growth and ewe health and also resulted in the most significant increase in both humoral and innate immune function in comparison to other Se sources (124). Chickens fed a diet containing Aflatoxin B1 developed aflatoxicosis and impaired humoral immunity, however Se supplemented chickens actively mitigated the immunotoxicity of aflatoxin B1 and this was concluded by studying the effects of AFB1 and Se in the primary lymphoid gland, Bursa of Fabricus. Authors stated that the ameliorative effect of Se on AFB1 induced immunotoxicity may be associated with reduced activation of mitochondria-mediated apoptotic pathway (125). Supranutritional Se-Y supplementation in ewes has been linked to an increase in immune response to parasitic infection (126). Studies have shown that antibody mediated immune response in lambs to *Pasteurella haemolytica* or *Pasteurella multocida* P52 antigen was significantly enhanced in Se treated groups (127, 128). Increased gene expression of L-selectin, IL-8R, and TLR-4 in neutrophils of sheep have also been associated with Se supplemented ewe diets, suggesting superior functioning of recognition and response to bacterial and parasitic threats (124).

1.4.3) Reproduction

Adequate Se intake is essential for the normal functioning of the reproductive system in both male and female animals. Se deficiency in males can lead to reproductive disorders and cause low fertility, poor sperm quality and abnormal sperm structure (111, 129, 130). Oxidative stress

can result in lipid peroxidation of spermatozoa and sperm plasma membrane which contains a high proportion of polyunsaturated fatty acids (PUFA). Se supplementation protected sperm plasma membrane from lipid peroxidation of PUFA, therefore improving sperm functional ability (131). Emerging evidence is showing that Se exerts an influence on the biosynthesis of testosterone, this is of great interest as sex hormones including testosterone are responsible for the process of spermatogenesis. In Leydig cells, Se supplementation resulted in enhanced expression of steroidogenic acute regulatory (StAR) and hydroxysteroid dehydrogenase 3β-HSD, these are the two main proteins that catalyse testosterone biosynthesis (132). Accumulating evidence suggests a role for Se in safeguarding the balance between germ cell death and proliferation during apoptosis in spermatogenesis, and therefore protecting male fertility (111). Recently a study exhibited using sheep Leydig cells, that Se can heighten testosterone production by activating the ERK signaling pathway and Se effects proliferation and apoptosis in Leydig cells hence showing that Se plays a crucial role in regulating and maintaining testosterone levels and spermatogenesis (132). Dietary Se supplementation in roosters was shown to regulate mRNA expression of apoptosis and cell cycle genes in the testis during spermatogenesis (133). GPx4 is inactive in mature spermatozoa however; it does exert its antioxidant activity against mitochondrial oxidative damage during spermatogenesis (134). Se supplementation increased gene expression levels of luteinizing hormone/choriogonadotropin receptor (LHCGR) in chicken testis, LHCGR regulates the action of lutenizing hormone on Leydig cell functions to support germ cell development and spermatogenesis (130).

Recently studies have begun to show the importance of Se in female fertility, Se restriction or deficiency has been associated with miscarriage and pre-term labour and effects cellular proliferation in follicles and reproductive tissues (135–137). *In vivo* mouse studies have shown that the uterus of Se deficient subjects is more prone to inflammation, it is thought that a Se

restricted diet heightens the activation of Toll-like receptor-4 signaling pathway which enables inflammatory gene expression such as tumor necrosis factor-α, interleukin-1β and interleukin-6 (138). To date, there has been relatively few studies investigating the effects of Se on female fertility, therefore the exact mechanism of Se on female fertility is not well understood. Only recently has there been an emergence of studies researching this topic. Granulosa cell proliferation and estradiol-17β has been proven to be modulated by Se supplementation (139, 140). One such study used X-Ray fluorescence imaging to identify cell specific accumulation of Se in bovine ovaries and determined that GPx1 is consistently amassed in the granulosa cells of large healthy follicles. Similar dispersal of GPx1 was not observed in smaller follicles, indicating that selenoprotein GPx1 plays a crucial role in follicle dominance (139). It has been reported that organic and inorganic Se has differing effects on female reproduction. Gene expression analysis studies revealed that gene transcripts connected with energy production and protein translation were increased in the oviduct of hens fed an Se-Y (Sel-Plex) supplemented diet but was not observed in subjects fed an inorganic Se diet (141). Like the male reproductive system, oxidative stress can negatively impact female fertility. Pre-ovulatory follicle-generated ROS are required for the induction of ovulation, however, an imbalance between prooxidants and antioxidants has been shown to result in female fertility disorders such as polycystic ovary syndrome, endometriosis and unexplained infertility (135, 142, 143). Recent evidence illustrates that low serum Se and GPx in follicular fluid is linked with non-fertilized oocytes, while diminished Se and GPx levels were significantly lower in subjects with endometriosis (135, 144). Maternal Se deficiency in pregnancy can lead to adverse pregnancy outcome and foetal development. High Se uptake by the developing foetal tissue occurs during pregnancy therefore increasing maternal Se requirements (145). Recent investigation indicates that Se supplementation during the pregestation and gestation periods results in the highest percent occurrence of good quality blastocysts. Se supplementation during

gestation is also believed to have an embryoprotective ability against the harmful effects of ROS activity (146).

1.5) Heavy Metal Contamination:

Animal feed contamination is a growing area of concern for the modern agricultural industry. The increasing world population has triggered a significant rise in the demand for food production and has placed immense pressure on agriculture and food production industries (147). This has led to an escalation in anthropogenic and industrial activities which in turn has resulted in higher emissions of environmental toxins (148). Animal exposure to environmental toxins can occur via soil, water, food or air and leads to poor animal health, increased food production costs and poses a potential risk to human health through bioaccumulation in the food chain (149). Commonly detected feed contaminants include heavy metals such as Arsenic (As), Lead (Pb) and Cd, dioxins and PCBs, pesticide residues and veterinary drugs (see Table 1.3) (150). The use of animal feeds in intensive farming requires supplementation with various additives such as vitamins, trace elements, growth promoters and antioxidants. The inclusion of several different additives poses a challenge to the aim of ensuring that all components are contaminant free and that possible residues do not pose a risk to human health (151). A recent study investigated the impact of the increased demand for intensive poultry farming and highlighted an increased risk to human health due to animal feed contamination. High levels of copper (Cu), zinc (Zn) and As were detected in chicken livers as a direct result of animal feed contamination, thus posing a potential risk to human health (152). Most feed contaminants cannot be metabolised and excreted by humans and animals, therefore these compounds accumulate in tissues. For example, PCBs are lipophilic compounds that when ingested, bioaccumulate in fat tissues, therefore rendered animal fats and oils routinely added to animal

feed represent a significant source of exposure to dioxins and PCBs, both for the animal directly and for humans on consumption (153).

Table 1.3: Common animal feed contaminants, sources and biological effects.

Feed Contaminant	Sources	Clinical Effects
1) <u>Heavy Metals</u>		
• **Cadmium**	Manure, Sewage, Phosphate Fertilizers and Mineral Supplements such as phosphate and zinc sources.	Nephrotoxicity, Neurotoxicity, Carcinogenicity, Osteoporosis, Genotoxicity, teratogenicity and endocrine and reproductive effects (149).
• **Arsenic**	Soil, Groundwater, Plants and Marine Organisms. Used as a feed additive in poultry and pig farming to increase body weight.	Effects the cardiovascular, reproductive, respiratory, gastrointestinal, hematological, and immune system (149).
• **Mercury**	Mining, smelting and industrial activities. It is deposited into soil and water where it is transformed into methylmercury and bioaccumulates in aquatic mammal tissues.	Affects the renal, nervous, gastrointestinal, reproductive, respiratory and musculoskeletal system. Mercury is embryocidal (149).
2) Environmental Contaminants		
• **PCB's / Dioxins**	Produced from the combustion processes and from industrial processes such as bleaching of paper pulp using chlorine.	Accumulation of PCB's and dioxins in animal and human tissues can lead to cancer and reproductive and immune dysfunction (154).
• **Organophosphate /Organochlorine**	Pesticide residues such as chlortetracycline, sulphonamides, penicillin and ionophores are the most commonly used	Metabolites of pesticide residues are stored as metabolites in the tissues and fluids of the animal causing concern for human health. Exposure can lead to cancer, infertility and liver disease (150)
• **Nitrate/Nitrite**	Nitrates can occur in various crops via the use of highly soluble mineral fertilizer Nitrate is converted endogenously to nitrite in either the saliva of monogastric mammals or stomach/rumen of ruminants	Results in cyanosis and death at high levels due to the formation of MetHb which impairs the ability of the blood to carry oxygen (150, 155).
• **Antibiotics**	Veterinary drugs including penicillin, tetracyclines,	Antimicrobial drug resistance, allergic reaction, carcinogenicity, mutagenicity, teratogenicity, disruption of intestinal flora and human antibiotic resistance (156).

The World Health Organisation deems heavy metals as the most hazardous xenobiotic for human health and lists As, Cd, Pb and mercury (Hg) among the ten major chemicals of public concern (157–159). Heavy metals occur naturally within the earth crust and as a result of weathering and volcanic eruption. However, the issue of environmental contamination and human exposure has arisen mainly from anthropogenic activities (157). Heavy metals produce their toxic effects by forming complex structures with thiol groups and by competing with and displacing biogenic metals (Zn, Cu, iron (Fe), calcium (Ca) etc.) to form metal complexes. This leads to inactivation and malfunction of many biological proteins and enzymes (160). Heavy metal toxicity is also mediated by the metals ability to modify cellular redox states thereby stimulating oxidative stress and initiation of the formation free radicals (160, 161). Presently, As, Pb, Hg and Cd are the most commonly detected heavy metal contaminants in the environment and pose a significant threat to human health by bioaccumulation through the food chain (149, 162, 163). Metalloid contamination is of grave concern in food production of animal origin as metals are readily transferred through food chains (164). Studies have been conducted to determine the prevalence and concentration of heavy metals in livestock feeds and animal manure, with results highlighting non permissible levels of As, Zn Cd and Pb in poultry, cattle and pig feeds (165–168).

The first possible mechanism of heavy metal induced oxidative stress is based on the production of free radicals. Heavy metals cannot directly generate reactive oxygen species; however, they indirectly cause oxidative stress by dislodging redox active metals from enzymes and proteins. The displaced ions can then partake in the generation of hydroxyl (OH) radical by the Fenton (Haber-Weiss) reaction (160). The second potential mechanism of heavy metal toxicity involves its ability inhibit cytosolic superoxide dismutase (SOD), GPx, and glutathione reductase (GR) by the displacement of Cu and Zn ions from the active centre of SOD. Heavy metal binding to reduced glutathione in the active site of GPx and GR efficiently inhibits these

antioxidant enzymes (169). Metalloid induced ROS cause numerous pro-mutagenic alterations to DNA bases, enhance lipid peroxidation and modify calcium and sulfhydryl homeostasis which support and expediate carcinogenesis (161). Heavy metals have carcinogenic potential that is principally reliant upon oxidation state, solubility, bioavailability and solubility of each individual metalloid (170). Metal induced carcinogenesis has also been associated with interference of zinc finger proteins and impairment of DNA repair systems (see Fig. 1.3) (160, 170).

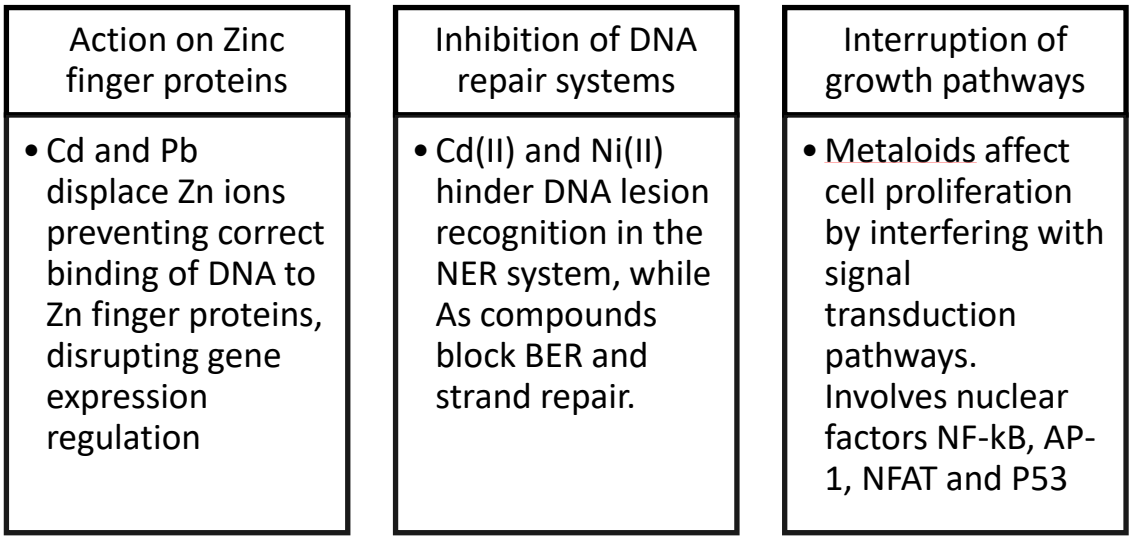

Figure 1.3: Mechanisms of heavy metal induced carcinogenesis (Adapted from (170))

1.5.1) Cadmium

Cd is a relatively abundant, highly toxic heavy metal which is regularly found in crops for food and feed production due to the highly soluble nature of Cd compounds. This ease of absorption of Cd from soil to crops makes the diet the primary source of human exposure to Cd (171). Cd absorption from the gastrointestinal tract is the main route of Cd exposure in humans. The International Agency for Research in Cancer (IARC) has classified Cd as a group 1 carcinogen while the European Protection Agency considers Cd as a probable human carcinogen (groupB1) (172). Cadmium contamination of animal feed is an increasing problem worldwide

with escalating levels of Rapid Alert System for Food and Feed (RASSF) alert notifications (see Table 1.4). Concerningly, none of these alert notices were classed as serious (173, 174).

Table 1.4: Rapid Alert System for Food & Feed (RASFF) Cadmium Contamination Notifications (174).

16/01/2014	**2014.0062**	**Belgium**	**cadmium (160 ppm) in poultry feed additive from Belgium**	**feed premixtures**
15/01/2014	2014.0057	Italy	cadmium (**2.81; 3.90 ppm**) in fish meal from Spain	feed materials
04/07/2014	2014.0924	Sweden	cadmium (**1.08 ppm**) in feed from the United Kingdom	compound feeds
03/08/2015	2015.1008	Germany	cadmium (**6.66 ppm**) in complete feed for dogs from Poland	compound feeds
23/09/2013	2013.1293	Germany	cadmium (**1.43 ppm**) in feed for cattle from Germany	feed materials
04/06/2013	2013.0777	Belgium	cadmium (**52 ppm**) in zinc oxide from Turkey, via Denmark	feed additives

Cd has a reported biological half life of approximately 20 to 35 years and accumulates primarily in the liver, lungs, pancreas and kidneys, there is no biological excretion pathway for the element (161). Cd officially belongs to the group of transition elements, however it almost always assumes only one oxidation state which is 2+, thus it behaves similar to main group metals (175). Like main group metals, Cd is unable to generate free radicals directly through

participation in the Fenton-like reaction, while the exact mechanism of Cd-induced oxidative stress is not yet known, indirect ROS generation has been reported in Cd exposed cell models (148, 160, 176). The factors underlying Cd toxicity include its ability to suppress mitochondrial functions, disrupt calcium homeostasis and modify cellular enzyme activity (177). Cd is capable of generating an environment of oxidative stress, triggering inflammation and endoplasmic reticulum stress, causes genomic instability and dyshomeostasis of essential metals (178).

Cd is absorbed preferentially in the duodenum and proximal jejunum following ingestion, therefore the gastrointestinal tract is one of the main areas affected by acute or chronic Cd exposure. It has been reported that up to 20% of dietary Cd is absorbed in the gastrointestinal tract, this quantity may escalate depending on the presence or absence of certain essential elements (Zn, Fe) (179). The Fe transporters divalent metal transporter 1 (DMT1) and FPN1 are responsible for Cd transport across the cell membrane (179). This suggests that Cd is transported across membranes by the same mechanism used to transport nonheme iron. Intestinal absorption of Cd in mammals increases in environments of Fe deficiency. Increased expression of the Cd transporter DMT1 was detected in duodenal mRNA (180) while enhanced levels of DMT1 and FPN1 were observed in Fe deficient mice which correlates with enhanced Cd absorption and accumulation (181). The mechanism of Cd transport and absorption across intestinal epithelia remains unclear. One study concluded that Cd transport occurs via transcellular transport exclusively, this mechanism involves passage across at least two membranes, is energy dependent and facilitated by membrane proteins (175, 182, 183). However, the requirement for tissue accumulation of Cd to obtain transfer of Cd across the intestine has also been illustrated (178, 184). Orally administered Cd resulted in haemorrhagic gastritis, epithelial cell necrosis and significant modifications in villi architecture in the mouse model (185). Cd stimulates metallothionein (Mt) production and binds with these cysteine rich

proteins to form a Cd-Mt complex. Mt has a known protective role in Cd induced toxicity, Mt's are metal binding proteins that detoxify and remove Cd from the cellular environment. However, they also scavenge Cd-induced free radicals which results in dissociation from Mt's caused by decreased metal binding stability. Mt's are also responsible for the retention of Cd in various tissues and the long biological half life of the metalloid in the body (171, 186). The protective role of Mt activity in Cd-induced oxidative stress has been illustrated *in vivo* using the mouse model. Mt null mice were hypersensitive to Cd toxicity, while Mt transgenic mice were mostly safeguarded from Cd induced damage (186). Unbound Cd and Cd-Mt accumulate in the kidneys due receptor mediated endocytosis uptake, the Cd-Mt complex is degraded in endosomes and lysosomes discharging free Cd into the cytosol which results in the production of Cd-induced ROS (163).

As mentioned previously, Cd is a redox-inactive metal and is incapable of directly generating ROS, instead it produces free radicals by indirect mechanisms. One proposed mechanism of Cd-induced oxidative stress is the displacement of Fe and Cu ions by Cd at cellular binding sites which leads to ROS generation via the Fenton reaction (see Fig. 1.4). In this process, unstable semiquinones are present due to the formation of $O_2^{\cdot -}$ during inhibition of complex III of the mitochondrial electron transport chain. This results in the generation of superoxide anion and superoxide anion derived ROS facilitated by the transfer of electrons to molecular oxygen by the unstable semiquinones (160).

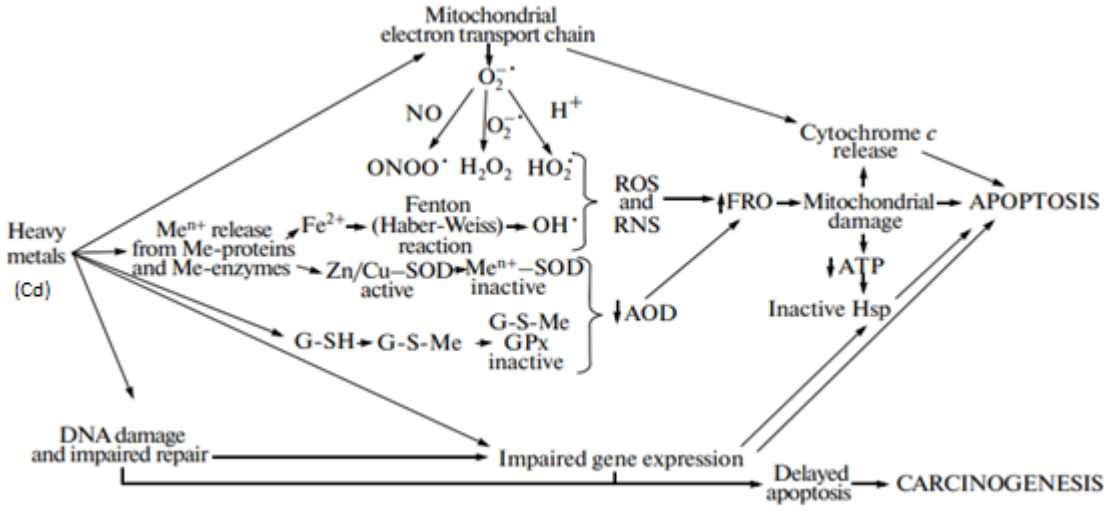

Figure 1.4: Mechanism of action of Cd and the induction of DNA damage, apoptosis and carcinogenesis (160).

The other mechanisms of Cd toxicity are as mentioned in Fig. 1.2 and include 1) action on Zn finger proteins 2) inhibition of DNA repair systems and 3) interruption of growth pathways. Cd exposure in three different types of human cell lines showed a significant increase in lactate dehydrogenase leakage, DNA damage, malondialdeyde and antioxidant enzymes activities. A reduction in ATP production was observed in HepG2 and HEK 293 cell lines and a decrease in GSH/GSSG ratio was reported following Cd exposure (187). The mitochondria are one of the main cellular targets of Cd toxicity, exposure of porcine kidney cell line (LLC-PK_1) to 20µM Cd illustrated that apoptosis occurred through the production of ROS, the breakdown of mitochondrial membrane potential and activation and release of caspase-9, -3 (80). Cd is capable of inducing apoptosis in various human cell lines and in the in vivo animal model (177, 187–189), the mitochondria is the primary cellular component involved in regulating apoptosis. The outer membrane of the mitochondrion is permeable to Ca^{2+} and regulates the flow of Ca^{2+} into the intermembrane space, Cd is believed to gain entry in the mitochondrion via Ca^{2+} uniporter. Inside the mitochondrion Cd attacks the thiol groups of adenine nucleotide translocator to form Cd-thiol complexes which disrupt many cellular functions (188). Cd has

been showed to cause opening of the mitochondrial permeability transition (MPT) pore which is linked to Cd induced formation of ROS at complex III (148). Opening of the MPT pore results in the leakage of harmful proteins that are essential for apoptosis, these include pro-caspases cytochrome c and Smac/Diablo. Apoptosis inducing factor is also released from the MPT pore and activates a group of nucleases that break up DNA into small fragments (188). Cd-induced oxidative stress was shown to trigger mitochondrial-caspase dependent apoptotic pathway in splenic and thymus cells in the mouse model (190) while in human embryonic kidney cells Cd-induced apoptosis was directly linked to structural and functional modifications to mitochondria caused by Cd exposure (188).

Free Cd mainly targets cellular GSH, depletion of GSH reserves leads to inefficient radical scavenging and causes distortion of the cellular redox balance (171). When GSH is depleted by Cd, several enzymes in the antioxidant defence system act to protect the cell from oxidative damage, however most of these enzymes are inactivated by the binding of Cd to the active site of the enzyme (191). Chronic and acute Cd intoxication effects the activities of enzymes, cellular components and lipid peroxidation in different ways (192). Oral administration of Cd in the rat model increased lipid peroxidation in liver mitochondria as well as increasing hepatic and renal peroxidation, a significant increase in GSH levels was also observed fifteen days after administration. However, rats exposed to a higher dose of Cd showed symptoms of strong acute toxicity within 24 hours which presented as severe hepatic damage, renal injury and abnormal serum electrolytes (193). Similarly, Cd can also modify the action of SOD, CAT, GR and GPx in various ways depending on the period of exposure (171). Upon short term exposure (4 hr) of liver cells to Cd all enzymes tested lost their enzymatic activities, however after longer exposure (8 hr) SOD and GPx activity levels increased while the action of CAT and GR decreased significantly (194). Analyses of the differential oxidation of nuclear and cytosolic TRx1 and mitochondrial TRx2 by Cd ions have highlighted a greater oxidation magnitude of

TRx2 compared to cytosolic TRx1 (114, 195). Oxidation of TRx by Cd resulted in the activation of Apoptosis signal-regulating kinase 1 (ASK1) and cell death, however apoptosis was not induced by oxidation of GSH illustrating that Cd oxidation of TRx and not GSH activates cell death (114).

1.6) DNA Damage and Repair

The living cell is constantly exposed to a variety of endogenous and exogenous DNA damaging agents which can culminate in genomic instability. Of the free radical species, reactive oxygen species (ROS) are an important group of DNA damaging agents and include the highly reactive OH radical, superoxide radical ($O_2^{\cdot-}$), singlet oxygen (O_2) and non-radical hydrogen peroxide (H_2O_2) (196). A major source of endogenous ROS production is mitochondrial based aerobic metabolism whereby, intracellular $O_2^{\cdot-}$ is generated by either NAPH oxidase enzymes which oxidize NADPH or by electrons leaking from the electron transfer chain in mitochondrial respiration. The superoxide anion radical ($O_2^{\cdot-}$) is produced by the one-electron reduction of molecular oxygen, it is a byproduct of metabolism and an essential component of immune defense (197). Leakage of electrons from the electron transport chain during oxidative phosphorylation is the main cause of $O_2^{\cdot-}$ generation with humans producing 5g of ROS per day through this mechanism (198). $O_2^{\cdot-}$ is cytotoxic and causes DNA damage, under normal circumstances a group of metalloenzymes the superoxide dismutases (SODs) preserve the optimal level of $O_2^{\cdot-}$ of the cell. SOD converts $O_2^{\cdot-}$ into H_2O_2, which is then converted into oxygen and water by GPx. However, $O_2^{\cdot-}$ that evades this defense mechanism can cause damage to proteins lipids and DNA. The H_2O_2 produced can initiate redox reactions by oxidizing protein cysteine residues, it has the ability to modify signaling pathways (P13/AKt) which stimulates cancer cell proliferation and has been associated with cardiovascular disease, diabetes and neurological disorders (199). Another way in which $O_2^{\cdot-}$ radical causes DNA

damage is by its participation in OH radical production. $O_2^{\cdot -}$ is converted by compartment-specific superoxide dismutases into H_2O_2 (200) which can be converted into highly reactive OH in the presence of ferrous ion via Fenton chemistry (201).

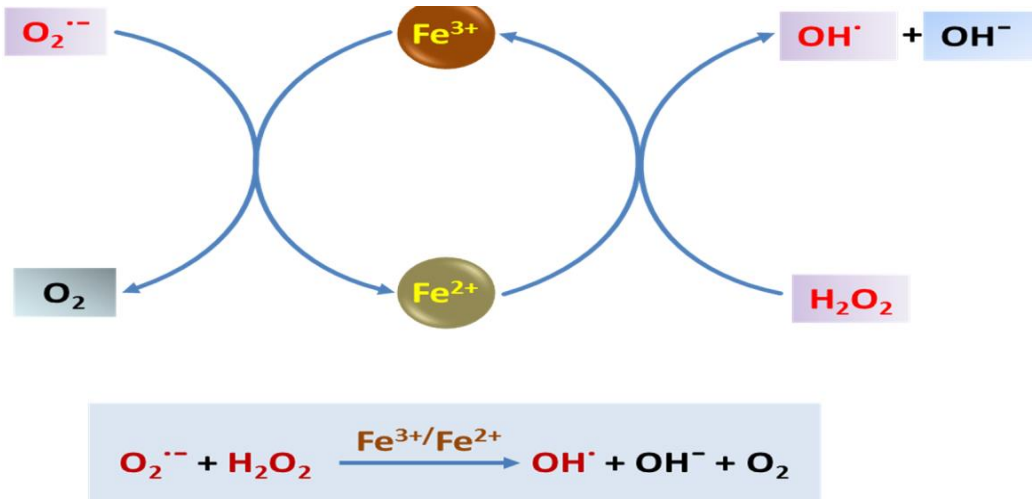

Figure 1.5: Iron catalysed Haber-Weiss reaction and Fenton reaction. $O_2^{\cdot -}$ and H_2O_2 react to form highly reactive OH and is referred to as the Haber Weiss reaction. in the presence of ferrous iron the reaction is accelerated by the presence of ferrous iron known as the Fenton reaction (202).

Irreversible damage to cellular macromolecules occurs when H_2O_2 concentrations in the cell increase exponentially and hydroxy radicals react with metal cations (Fe^{2+}) (200). The damaging oxidizing effects of $O_2^{\cdot -}$ were observed in SOD1 deficient mice where a lack of SOD1 resulted in cognitive impairment and neuronal dysfunction facilitated by oxidative damage of brain cells (203). Deficiency of manganese superoxide (SOD2) was shown to reduce protection against $O_2^{\cdot -}$ formation and damage, resulting in increased mitochondrial dysfunction and DNA fragmentation caused by the enhanced levels of mitochondrial oxidant stress (204). Fenton chemistry, namely iron-catalysed Haber-Weiss reaction is the mechanism by which the highly reactive OH radical is produced in biological systems (see Fig.1.5) (205) and its formation involves $O_2^{\cdot -}$ and H_2O_2 as stated above. Hydroxyl radical is known to add to the double bond of the four main purine and pyrimidine bases at diffusion-controlled rates. Hydroxyl radical preferentially adds to the site with the highest electron density due to its

electrophilic nature, therefore, molecules with high electron density will be more susceptible to OH attack (196). Degradation of nucleobases is characteristic of OH radical damage along with oligonucleotide strand breaks, DNA-protein cross-links and abasic sites (206). Hydroxyl radical modification of DNA has been linked with metastatic breast cancer progression (207), OH activity was also implicated in the formation of DNA-protein crosslinks (DPCs) in hybridoma cells exposed to Fe(II) which may have detrimental biological effects (208).

Oxidative stress occurs when levels of these ROS exceed the antioxidant defence capacity of the cell. Proteins lipids and DNA are the main cellular targets for ROS and can result in oxidised bases and DNA lesions, carbonylated proteins and lipid hydroperoxides (209). The DNA lesions that can occur as a result of ROS attack include; double strand breaks, abasic sites, modified bases, mismatched pairing and interstrand/intrastrand crosslinks. Double strand DNA breaks (DSBs) are highly cytotoxic lesions and are generated by extrinsic exposure to environmental toxicants or intrinsically by free radicals produced during cell metabolism. DSBs are lesions where the phosphate backbones of the two complimentary DNA strands are broken simultaneously (210). In the absence of repair, mutations and genomic instability can arise through the production of dicentric or acentric chromosomal fragments due to the presence of DSBs (211), genomic rearrangements can occur when multiple DSBs in the same cells are annealed erroneously. These lesions are highly toxic and may have tumorigenic potential (212). Efficient repair of DSBs is often limited due to their physical separation and damage endured at DNA termini that require processing. DSBs are repaired by either homologous recombination (HR) (see Fig. 1.6A) or non-homologous end-joining (NHEJ) (see Fig. 1.6B). Homologous recombination is essential for the maintenance of genome stability and avoidance of cancer development, it removes DSB lesions and preserves replication forks, telomere maintenance, and chromosome segregation in meiosis I. Homologous repair is an error free pathway which is mediated by eukaryotic recombinases Rad51 and Dmc1 (213).

Rad51 is one of the main proteins which catalyses homology search and DNA strand invasion, the two key reactions that categorize HR. The mechanism of HR is comprised of three core phases namely, presynapsis, synapsis and postsynapsis. During the first and second steps, DSB ends are identified and converted to a 3'-OH ending single stranded tail, the third step involves the generation of a D-loop by DNA strand invasion by the Rad51-ss DNA filament (214). DSBs have been associated with distinct ageing phenotypes in mouse liver characterised by fused mitochondria and alterations in gene expression profiles, it was shown that DSBs can expediate some features of the normal ageing process (212). Non-homologous end-join (NHEJ) is the second mechanism for DSB repair, it has no requirement for homology and directly ligates fragmented ends of DNA DSBs. The mechanism of repair can be divided into three steps; The first step in NHEJ repair is the DNA end identification and stabilization of the NHEJ complex at the DNA double strand break. The DNA ends are bridged, and the DNA ends are processed. Fragmented ends of the DNA DSB are ligated and the NHEJ complex is dissolved in the final phase of NHEJ repair (215). Evidence suggests that the NHEJ repair process is inherently error prone and frequently causes deletions, insertions and translocations (212, 216, 217).

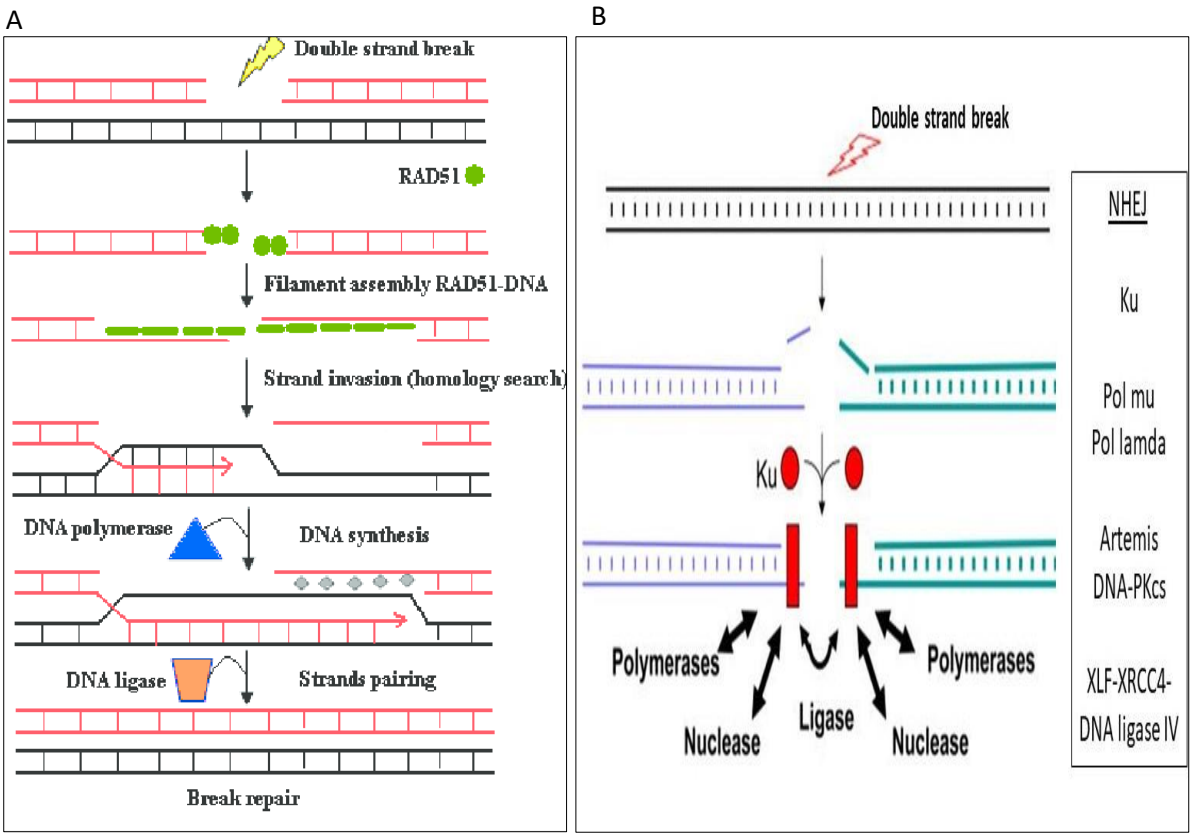

Figure 1.6: Mechanism of homologous recombination repair (A) and non-homologous end joining repair pathway (B).

DNA mismatch (MM) is another type of DNA damage that occurs when two non-complimentary bases are aligned in the same base pair step of double stranded DNA. MM is not well tolerated in DNA and can give rise to DNA lesions and genetic mutations if not accurately identified and repaired by MM repair proteins. Non complementary pairing of purine:pyrimidine are referred to as transduction, while noncomplimentary purine:purine and pyrimidine:pyrimidine pairing is defined as transversion (218). MMs that arise due to base modification require excision of the irregular base irrespective of the DNA strand it is located in. Currently researchers theorise that replication errors are caused by mismatch pairing of a non-tautomeric nature either by different chemical forms of bases (e.g., bases with an extra proton) or between bases that bond incorrectly due to a minor shift in position of the nucleotides. This type of mismatched pairing is known as wobble (219). Insertion/deletion

(IDL) mismatches are another type of MM and can be a result of polymerase-induced errors or exposure to carcinogens or irradiation. IDL mismatches are characterised by one or more extra base on one strand of the DNA than the other. Translesion synthesis may incorporate a nucleotide opposite the noninformational site, and this can introduce MMs and protuberances (220). DNA MMs induced by replication error, environmental toxins, spontaneous changes to DNA prior to replication and chemical reactions within the cell can be identified and corrected by DNA repair process. If the MM is not detected and amended, the extra base or incorrect base pair incorporated into the newly synthesised strand may become a permanent mutation (219).

To preserve the stability and integrity of the genome, the cell has acquired several mechanisms to repair DNA damage reduce the risk of disease by removing deleterious lesions. The repair mechanism utilised by the cell to correct MM is the mismatch repair pathway (MMR) (221). The function of MMR is to mend replication errors in newly created DNA and serves to prevent homologous recombination between DNA sequences. Defects in the MMR system has been shown to increase the incidence of sporadic and hereditary cancers as the spontaneous mutation rate is amplified (222–224). Mis-incorporation of nucleotides cause genetic changes to DNA and do not generally generate changes in DNA structure and therefore are more difficult for the cell to detect. Hence, there is a group of specialised mismatch binding factors for MM recognition and coordination of nucleolytic excision which include; homologs of the bacterial MutS (e. g. human MSH2, MSH3, MSH6) and MutL proteins (e. g. human MLH1, PMS2). The mechanism of MMR is best understood in *Escherichia coli*, where MutS, MutL and MutH are required to initiate MMR and have crucial biological roles in MMR. Small IDL, base-base MMs are initially detected by MutS, the binding of MutS to the DNA mismatch as a homodimer is the first step in the MMR pathway (225). MM recognition is enhanced by the interaction of MutL with MutS, which triggers the recruitment and activation of MutH. The unmethylated

daughter strand of hemimethylated dGATC is incised by MutH and this strand-specific nick provides the initiation site for mismatch-provoked excision. Helicase II, in the presence of MutL unwinds the duplex from the nick towards the MM, producing a single strand of DNA (226). The nick is excised from the strand by ExoI or ExoX (3′→5′ exonuclease), or ExoVII or RecJ (5′→3′ exonuclease) slightly passed the MM location. Finally the single stranded gap is repaired by DNA resynthesis and ligation by DNA polymerase III holoenzyme, SSB (single-stranded DNA binding protein), and DNA ligase(221). MMR pathway may face competition from other repair activities within the cell, mammalian DNA glycosylases such as Thymine-DNA glycosylase may compete for G T and Adenine-DNA glycosylase MutY for G A or C A mismatch binding. To enable efficient and accurate DNA repair and to avoid generation of DNA mutations, coordination between the various repair mechanism is critical and can be accomplished by spatiotemporal regulation of MM recognition (227).

DNA crosslinks are formed due to covalent bond formation between two nucleotide residues from the same DNA strand (intra strand) or from opposite strands (inter strand (ICL) (228). While intra strand crosslinks can be efficiently corrected by nucleotide excision repair (NER), an ICL obstructs DNA strand separation and blocks replication and transcription. The presence of ICLs can greatly distort the structure of DNA, therefore if left unrepaired ICLs are highly cytotoxic and lead to cell death (229). Crosslink formation requires two independently reactive groups in a single alkylating molecule, an ICL is formed when the two reactive groups react with two bases on opposing sides of a DNA strand (230). Crosslinking agents exist as synthetic compounds and occur naturally in the environment due to cellular metabolic processes. Direct evidence and understanding of endogenously generated ICLs is scarce since they are infrequently formed by living cells (231). However, studies have been conducted to simulate ICL production in the cell by treating oligonucleotides with relevant ICL inducing agents or by analysis of the mutations introduced into reporter cells (231, 232). It was observed that

malonic dialdehyde a by-product of lipid peroxidation induced the formation of ICLs. Similar DNA adducts were identified in cells exposed to unsaturated aldehydes of environmental origin including; acrolein, crotonaldehyde, while the condensation reactions of formaldehyde and acetaldehyde with DNA also produced adducts (233, 234). The main motivation for analysing ICLs has been there importance as adducts formed by anticancer agents as outlined in Table 1.5.

Table 1.5: ICL inducing anticancer agents. Adapted from (235).

Drug	Mode of Action	Major side effects
Cisplatin	DNA crosslinker	Nephrotoxicity, neurotoxicity, ototoxicity
Carboplatin	DNA crosslinker	Myelosuppression
Oxaliplatin	DNA crosslinker	Neurotoxicity, pulmonary toxicity, hepatotoxicity

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Cisplatin is one of the most commonly used anticancer therapeutics and treats a broad array of cancers including ovarian, breast, cervical, bladder and oesophageal cancers. The compound contains a platinum core with two chloride leaving groups and two amine non leaving groups. After the compound enters the cell the platinum binds to the guanine residues and adenine residues forming a DNA adduct. An intra strand crosslink is generated when two platinum adducts are formed on adjacent bases (235). Unrepaired or misrepaired ICLs cause genome instability and block DNA replication and transcription by preventing DNA strand separation. Repair of ICLs is performed by either replication dependent or replication independent repair. Bulky ICLs that distort DNA structure are repaired through both pathways, while non-distorting DNA adducts are corrected primarily through replication dependent repair.

1.6.1) Base Excision Repair

Base excision repair (BER) is responsible for the correction of small lesions which do not alter DNA structure but introduce genetic changes that lead to lethal mutations, BER repairs lesions generated by oxidation, deamination, alkylation and spontaneous DNA decay. BER aids in maintaining the chemical integrity of DNA under conditions of oxidative stress caused by endogenous or exogenous sources (236, 237). BER has a critical role in the detection and repair of highly mutagenic lesions such as 8-oxo-guanine (8-oxo-g), FapyG, Sp and GH (236), There are several variations in the BER theme, however, the foundations of each pathway are similar and generally incite the four steps illustrated in Fig. 1. 7 (238).

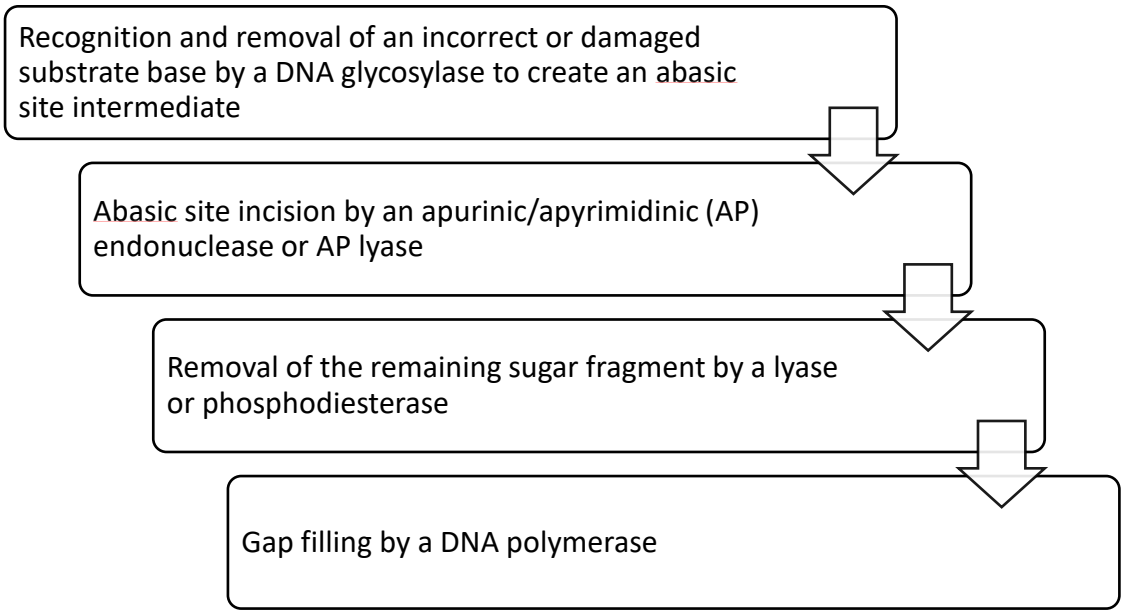

Figure 1.7: Four phases of typical Base Excision Repair (238).

The first step of the BER pathway is initiated by DNA glycosylases which detect the damaged base and cleave the N-glycosylic bond between deoxyribose and the target base freeing a base and leaving an apurinic/apyrimidinic site (239). Currently, 11 mammalian glycosylases are

known (see Table 1.6), with most recognizing several different lesion with frequent overlapping of specificity (237). DNA glycosylases are described as either monofunctional or bifunctional according to their catalytic mechanism and the way in which they cleave the DNA strand. Monofunctional glycosylases produce an abasic site by using a water molecule as a nucleophile to attack the aromatic carbon of the damaged base thereby stimulating base release (238). All monofunctional glycosylases possess only glycosylase activity, all mammalian glycosylases that are selective for uracil are monofunctional, other examples include MPG and MUTYH which correct A that is mispaired to 8-oxo-G, G or C (240). The bifunctional glycosylases excise damaged bases by using an active site amine molecule as a nucleophile, and during the catalytic process create a covalent Schiff base protein-DNA intermediate (238). In addition to glycosylase activity, this class of DNA glycosylase exhibit a β-lyase activity that cleaves the phosphodiester backbone by β elimination (NTHL1) or by two consecutive elimination steps β,δ elimination (NEIL1) leaving 3' ends that require further processing before BER (237, 238). Many DNA glycosylases involved in oxidized base excision are bifunctional.

Table 1.6: Mammalian glycosylases and the effect of gene knockout/knockdown (237).

Name	Type of Lesion	Mono/Bi functional	Mutant phenotypes – Mouse knockout (Ko)/knockdown (Kd)
UNG **SMUG1**	Uracilin ssDNA/ds DNA	Monofunctional Monofunctional	Ko: viable, B-cell lymphomas, disturbed antibody diversification kd: moderate increase in mutation frequency (C→T)
MBD4 **TDG**	Pyrimidine derivates in mismatches	Monofunctional Monofunctional	ko: viable, elevated mutation frequency (C→T) Ko: embryonic lethal, aberrant DNA methylation and imbalanced chromatin marks in CpG-rich promoters
OGG1 **MYH**	Oxidative base damage	Bifunctional Monofunctional	ko: viable, accumulation of 8-oxoG, elevated mutation frequency (G→T) Ko: see OGG1
MPG	Alkylated purines	Monofunctional	ko: viable, elevated levels of ethenoA and hypoxanthine
NTHL1 **NEIL1**	Oxidized ring fragments or saturated pyrimidines		Ko: viable ko: metabolic syndrome, increased damage levels in mitochondrial DNA; kd: hypersensitive to γ radiation

Following initiation of BER by specific DNA glycosylation the BER pathway will follow one of two subpathways, either short-patch repair (single nucleotide BER) or long patch repair (two or more nucleotides) (see Fig. 1.8). Short patch (SP) repair is largely the dominant subpathway in BER with long patch (LP) repair only being employed in post replicative repair. The major core proteins recruited in the SP pathway include AP-endonuclease APE1, DNA polymerase β (Pol β), and DNA ligase I or III (237). Polβ is the repair synthesis polymerase of the SP pathway

which typically introduces only one nucleotide, Polβ likely incorporates the first nucleotide in LP repair, however the elongation step in LP utilises replicative DNA polymerases (241). Polβ used in SP repair also contains dRPase activity that releases the 5' dRP end and allows DNA ligation (242). SP is an efficient repair system that requires several specific proteins that are not involved in the replication process and is equivalently proficient in both proliferating and nonproliferating cells. The LP pathway mainly occurs in proliferating cells where DNA synthesis is performed by replicative polymerases δ or ε function to displace the strand containing the 5'-dRP terminus and create a 'flap' structure that is excised by the flap endonuclease FEN1 to generate a ligatable substrate (242, 243). During repair synthesis the LP pathway introduces multiple nucleotides (2 to 12) the polymerases work in conjunction with the accessory 'clamp' protein, proliferating cell nuclear antigen (PCNA) (244). Pathway selection is dependent on cell type, ATP levels, protein-protein interactions, type of lesion, availability of BER factors, cell stage and whether the cell is actively dividing or terminally differentiated (237, 238). Lesions such as C1'-oxidized abasic lesion 2-deoxyribonolactone are resistant to Polβ activity and must be processed by LP repair. Error prone translesion polymerases (Polι, Polη, and Polκ) occur infrequently in the BER pathway however, due to their low fidelity any participation in BER synthesis would be highly mutagenic. To prohibit the introduction of mutagenic lesion formed by these error prone polymerases, the 5 dRP covalently traps the polymerases and inhibits their activity during DNA synthesis, the polymerases form Schiff formations without releasing the 5 dRP thereby, preventing the involvement of theses error prone pols and avoiding the high incidence of mutagenesis and carcinogenesis (245).

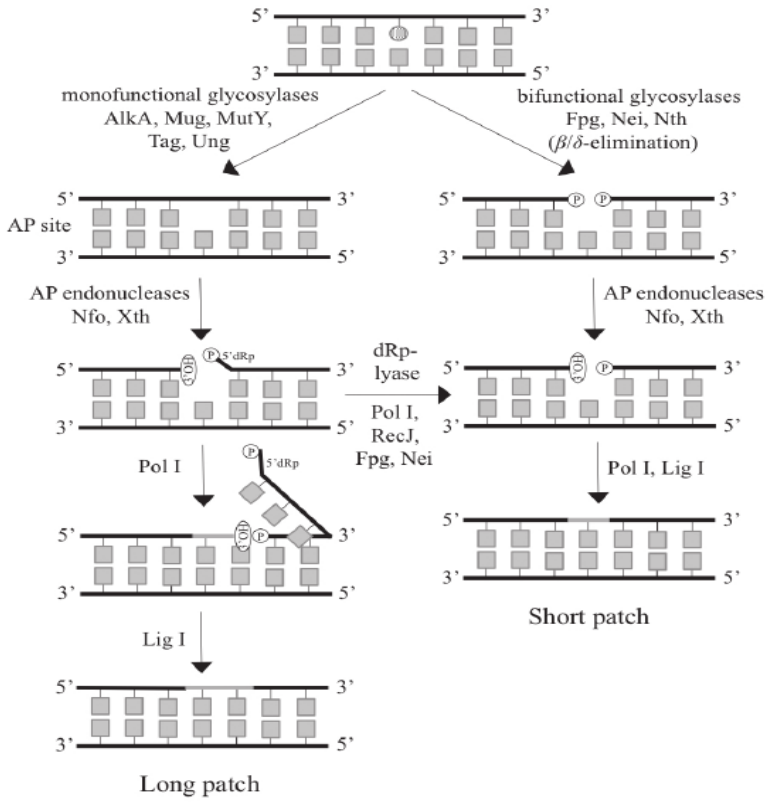

Figure 1.8: Long-patch and short-patch BER pathways. The first step in BER is the recognition and removal of base lesions (stippled circle). Monofunctional glycosylases create AP sites, which are cleaved at their 5′-side by an AP endonuclease leaving a 3′-hydroxyl (3′-OH) group and a 5′-deoxyribose phosphate (5′-dRp) terminus. Bifunctional glycosylases display AP lyase activity, with a β- or β/δ elimination activity and involves the generation of a single nucleotide gap containing 5′- and 3′-phosphate (P) groups at the termini. In the long-patch pathway, the 5′-dRp and 2–10 nucleotide patches are replaced during strand displacement by Pol I and subsequently ligated. However, in the short-patch pathway mediated by monofunctional glycosylases, the 5′-dRp terminus is removed by Fpg, Nei, RecJ or by the exonuclease activity of Pol I, generating a 5′-phosphate terminus. In the short-patch pathway involving bifunctional glycosylases, the 3′-phosphatase/phosphodiesterase activity of AP endonucleases will remove the corresponding blocking phosphate. Gap-filling and ligation steps then take place (264).

BER is the major pathway tasked with the repair of oxidatively induced DNA damage, all four nucleobases are susceptible to attack from ROS with guanine being the most vulnerable and leads to several oxidized guanine products. One of the most common and extensively studied guanine oxidation products is 7,8 dihydro-8-oxoguanine (8-oxoG) and occurs by the incorporation of an oxo group to the carbon at the C8 position and a hydrogen atom on the nitrogen position at 7 (246). 8-oxoG can simulate T functionality in the *syn* conformation,

resulting in a stable 8-oxoG(syn)A(anti) base pair thereby increasing the deleterious nature of the lesion. The harmful effects of this 8-oxoG(syn)A(Anti) were observed in a series of X ray crystal structures of DNA polymerase I fragment from *Bacillus stearothermophilus* (247). The presence of 8-oxoG(syn)A(Anti) was observed to induce template and polymerase distortions during replication which led to G-to-T transverse mutations, therefore 8-oxoG must be removed prior to replication (236). OGG1 is the main DNA glycosylase that removes 8-oxoG lesions, it is a bifunctional glycosylase that initiates BER and excises the damaged base(s) (248). Following base excision OGG1 cleaves the phosphodiester bond by utilizing its AP lyase activity, OGG1 initiated BER involves several steps including lesion recognition, flipping of the substrate from the DNA strand into the active site of OGG1 (249). The mammalian glycosylases OGG1, MTH1 and MUTYH play an important role in the removal of 8-oxoG lesions, MTH1 hydrolyses 8-oxodGTP eliminating it from the nucleotide pool to prevent its incorporation into DNA (250). Excision of 8-oxoG from the 8-oxoG-C base pair is performed by OGG1 allowing for further downstream processing by BER enzymes that can restore the G-C base pair. However, if this does not occur an incorrect 8-oxoG-A base pair will be formed, MUTYH must then intercept and remove the incorrect A base. An 8-oxoG-C substrate for OGG1 may then be generated by further processing of the AP site and subsequent replication by a DNA repair polymerase (236).

In contrast to the positive observations on the role of the OGG1-BER pathway, a recent study has observed that OGG1-initiated BER amplified oxidative stress induced cell death. The study illustrated that overactivation of PARP1 (the DNA damage sensor) is linked with apoptosis-inducing factor (AIF) and caspase-independent cell death known as parthanatos. The production of repair intermediates (e.g. APE1) that increased cellular levels of ROS induced DNA damage was observed to be the cause of PARP1 overactivation. Variants or deficiency of the DNA glycosylase MUTYH has been shown to have detrimental effects on the cell. A

direct connection has been established between colorectal cancer and mutations in the gene encoding human MUTYH glycosylase known as MUHYT associated polyposis (MAP) (236). Germline mutations in the NTHL1 which is involved in BER of oxidized pyrimidines is also linked with a polyposis similar to MAP (251). Whole exome sequence analysis of the G:C>T:A revealed a specific mutational signature (signature 36) caused by unrepaired 8-oxoG lesions. Interestingly it was observed that signature 36 strongly resembled the mutational signature discovered in neuroblastoma known as signature 18, this signature has also been found at lower levels in breast, pancreas and gastric cancers. It can therefore be hypothesized that oxidative DNA damage influences cancer aetiology in these organs (252).

While 8-oxoG has been the most extensively studied lesion to date, many other oxidized guanine products have been identified. G-to-T and G-to-C transversions are the most common cellular mutations the former can now be explained by the presence of 8-oxoG or FapyG while the latter is still unresolved. It is hypothesized that G-to-C transversions are mediated by other oxidized lesions, two hydantoin products have been highlighted as important lesions of interest; spiroiminodihydantoin (Sp) and 5-guanidinohydantoin (Gh) (236). These two lesions arise due to further oxidation of 8-oxoG (240) and have been proven to be highly mutagenic in both *in vitro* and *in vivo* (253). Singlet oxygen, high valent metals such as Cr(V) and Ir(IV) and ionizing radiation readily oxidize 8-oxoG to generate Sp, Gh and its isomer Ia (iminoallantoin) (254). Sp lesions occur primarily when the substrate is a nucleoside or single stranded DNA whereas Gh is mainly located in double stranded DNA (255). Unlike 8-oxoG which is mildly mutagenic (3%), the two hydantoin lesions are 100 percent mutagenic and mediate both G-to-T and G-to-C transversions. Sp strongly blocked replication in E.coli based mutagenicity assays, whereas the Gh lesion was more readily bypassed in E.coli and human studies due to its structure flexibility (256, 257). Observations of the flexibility of Gh offered further support to the hypothesis that flexible DNA lesions that present less of a barrier to transcription and

replication, cause increased transcriptional mutagenesis and are less vulnerable to removal by transcription-coupled DNA repair (253). A strong connection between infection induced colitis and the presence of Sp and Gh lesions was reported in the mouse model and may eventually lead to initiation of colon cancer (258). Sp and Gh lesions are removed by BER, the Nei-like (NEIL) family of enzymes are responsible for repair of these lesions and consists of NEIL1, NEIL2 and NEIL3 (254).

NEIL1 and NEIL2 are a bifunctional glycosylases that cleave the phosphodiester backbone by two consecutive elimination steps β,δ elimination leaving 3' ends that require further processing by BER enzymes. Proper ends for the DNA polymerase to act on are provided by polynucleotide kinase (PNK) instead of the usual APE enzyme (259). NEIL1 has several substrates including 5-hydroxyuracil, Fapy nucleotides and thymine glycol and exhibits limited activity towards 8-oxoG whereas NEIL2 displays affinity for certain oxidized products of pyrimidines (236). Gh and Sp are the best substrates identified for NEIL1/2 glycosylases thus far. NEIL1/2 operate on single stranded DNA, bubble DNA (single stranded DNA flanked by duplex regions) and bulge DNA but at a much slower rate than canonical DNA. Evidence suggests that NEIL1 does not require a base opposite the lesion to identify and remove hydantoin lesions. Results have indicated that NEIL1 activity may impede replication or result in the incorrect removal of bases which may ultimately increase potential mutagenicity (259, 260). Unlike NEIL1/2, the third Nei-like glycosylase NEIL3 is less well characterized. In contrast to NEIL1/2 that use proline as the nucleophile, NEIL3 possesses an N-terminal valine and forms a Schiff base intermediate (261). Murine NEIL3 has a preference for single stranded DNA and bubble DNA, whereas *E.coli* NEIL3 displayed a lower level of nicking activity at the AP site in comparison to NEIL1 (261, 262). Human NEIL3 acts as a monofunctional glycosylase and excises Sp and Gh in single stranded and double stranded DNA, it incises the DNA strand by β elimination only. It also exhibited efficient removal of 5-hydroxy-2'-

deoxycytidine (5OHC) and 5- hydroxy-2'-deoxyuridine (5OHU) in single stranded DNA (263).

The objective of the present study was to evaluate the potential effects of a range of organic and inorganic Se sources on Cd-induced toxicity in a porcine gut epithelial cell model. To elucidate a potential mechanism of action of Se on Cd-induced damage, antioxidant activity, apoptosis-associated DNA fragmentation, DNA repair capacity and transcriptome analysis of the three Se-Y compounds were performed. The study conducted in chapter two was the first study to directly compared the effects of inorganic and organic Se in a porcine gut epithelial model of Cd induced DNA damage. The results presented in chapter 3 highlighted important differences in the bio-efficiency of three different commercially available Se-Y preparations in terms of their ability to enhance a range of cellular mechanisms including DNA repair and antioxidant defense which protect porcine gut epithelial cells from damage due to Cd exposure. The transcriptomic analysis performed in chapter 4 details the RNA sequencing technology and transcriptomic analysis to detect differential gene expression in porcine jejunal cells following Se pre-treatment and Cd exposure. Overall the data showed that organic Se had a significant protective effect including an enhancement of cell viability and a reduction in the extent of DNA damage whereas inorganic Se potentiated the deleterious effect of Cd in this context. The results also highlighted the variation in the ability of different commercially available Se-Y preparations to protect porcine gut epithelial cells from Cd-induced damage.

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Chapter Two: Selenium source impacts protection of porcine jejunal epithelial cells from cadmium-induced DNA damage, with maximum protection exhibited with yeast-derived selenium compounds.

Abstract

Selenium (Se) is found in inorganic and organic forms, both of which are commonly used in animal feed supplements. The aim of this study was to determine the impact of the chemical form of Se on its associated ameliorative effects on cadmium (Cd) induced DNA damage in a porcine model. At a cellular level Cd mediates free oxygen radical production leading in particular to DNA damage, with consequential mutagenesis and inhibition of DNA replication. In this study, porcine jejunal epithelial cells (IPEC-J2) were pre-incubated for 48 h with one of Se-yeast (Sel-plex), selenomethionine (Se-M), sodium selenite (Se-Ni) or sodium selenate (Se-Na). The effects of this supplementation on cell viability and DNA damage following cadmium chloride ($CdCl_2$) exposure were subsequently evaluated. IPEC-J2 cells were cultivated throughout in medium supplemented with porcine serum to generate a superior model that recapitulated the porcine gut epithelium. The results illustrated that Se antioxidant effects were both composition- and dose-dependent as evident from cell viability (Alamar Blue and 5-carboxyfluorescein diacetate acetoxymethyl ester) and DNA damage assays (Comet and TUNEL). Both the Se-yeast and Se-M organic species, when used at European Food Safety Authority guideline levels, had a protective effect against Cd-induced DNA damage in the IPEC-J2 model system whereas for inorganic Se-Ni and Se-Na sources no protective effects were observed and in fact these were shown to enhance the negative effects of Cd-induced DNA damage. It can be concluded that nutritional supplementation with organoselenium may protect porcine gut integrity from damage induced by Cd.

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2.1) Introduction

Cadmium (Cd) contamination of animal feed has become a major problem worldwide and is of concern due to its toxic effect and the potential bioaccumulation of Cd through the food chain. Cd is a heavy metal which is naturally present in the environment due to volcanic emissions and the weathering of rocks. However, in recent decades increased Cd levels have become a concern, primarily due to anthropogenic and industrial activities, the result of which includes contaminated animal feed and feed supplements (1). The spreading of animal manure has also been shown to contribute to increased levels of Cd in soil, caused directly by contaminated animal feed products (2). The main route of Cd exposure is through the consumption of contaminated food and water. The European Food Safety Authority (EFSA) has set out regulations regarding permissible levels of Cd in food for human consumption, however there are currently no regulations relating to acceptable levels of Cd in animal feed. The human body has limited defence mechanisms against Cd, resulting in its bioaccumulation in the food chain. Cd has been shown to induce oxidative stress at a cellular level, with a positive correlation noted between Cd dose and the extent of DNA damage (3). The generation of Cd-induced free radicals has multiple effects on the cell including DNA strand breakage, the generation of mutations and the inhibition of DNA synthesis itself. Cd also affects cell proliferation, cell cycle progression, DNA repair mechanisms, cell differentiation and has also

been shown to modify apoptotic pathways (reviewed by (4)). Cd exposure has been reported to lead to nephrotoxicity, osteoporosis and neurotoxicity (5) and to adversely affect gut health and integrity in rats. The gastrointestinal tract is a primary target for Cd-induced damage (6) and a recent study observed tissue injury and a compromised intestinal barrier following Cd exposure (7).

Selenium (Se) is an essential trace element that possesses antioxidant properties and can protect the cell against the harmful effects of reactive oxygen species (ROS), thereby protecting DNA from oxidative damage and consequential disease (8). Se is a vital dietary component for both humans and animals as it is necessary for the activity of selenoproteins such as glutathione peroxidases (GSH-Px) and thioredoxin reductases, both of which play important roles in detoxification and the function of antioxidants (9). GSH-Px is responsible for the regulation of hydrogen peroxide levels in the cell. Its activity is significantly influenced by the Se status of the body, suggesting that a decline in Se levels may induce a decline in antioxidant capacity (10). Se deficiency can lead to poor immune function and increased susceptibility to the damaging effects of ROS, thus promoting cognitive decline and increased risk of mortality in humans. In the Agri-Food industry, low Se status in animals is of great importance as it can lead to white muscle disease, poor reproductive performance and an inability to thrive (11, 12). Se can exist in various chemical forms, including organic selenomethionine (Se-M) or inorganic selenites and selenates, all of which are commonly utilised as feed additives. Organic and inorganic forms of Se are not metabolized alike (13). Inorganic selenate is reduced to selenite, which is then non-enzymatically reduced via production of selenodiglutathione (GS-Se-SG) to selenide. Selenide is prone to forming complexes with other feed components leading to the formation of insoluble, unabsorbable complexes which are then excreted, thus significantly reducing Se absorption and bioavailability. The metabolism of organic Se differs to that of inorganic varieties; organic Se compounds are metabolised by an enzymatic process

whereby dietary Se is incorporated into protein (9). One investigation concluded that 98% of Se from organic sources is absorbed compared to only 84% for inorganic Se (14). Several studies have demonstrated that humans absorb and retain Se from organic Se sources more effectively than from inorganic Se compounds ((9) and references therein). Se supplementation has been shown to decrease the risk of prostate, lung, colorectal and bladder cancers due to its protective effect on oxidative DNA damage (15).

Se has been demonstrated to protect against Cd-induced damage in animal models, though results to date are ambiguous. Selenite inhibited Cd-induced damage in chicken kidneys when their feed was co-supplemented with both compounds (16) and the same effect was observed for Cd-induced damage in male rat livers with co-supplementation of selenite and Cd (17). In an in vitro study using rat hepatocytes, protective effects of selenite were shown to be dependent on Cd concentration and the selenite/Cd ratio, with no inhibition of DNA damage being seen at lower Cd concentrations (18). Another study showed that a combination of antioxidants, which included Se, had a protective effect against Cd toxicity in rat small intestine (19). Elsewhere, single supplementation with either Cd or selenite was cytotoxic and genotoxic for rats receiving high pharmacological doses (17) and this effect was even more pronounced for female rats (20). The potential toxicity of selenite in these models may be problematic, as Se is still widely employed as an animal feedstuff supplement (21). In pig nutrition in particular, the chemical form of Se has been shown to impact on the animal's growth and overall health (22). Additionally, organic Se was reported to be superior to selenite regarding the modulation of DNA repair pathways following lead-induced damage in human liver carcinoma cells (8). In the case of Se in particular, limitations have been demonstrated regarding the extrapolation of rat models to other models, meaning that the results observed in rat studies cannot automatically be applied to porcine models (23). It is clear that Se has a role in protecting against DNA damage, however, further work is needed to elucidate this role (24).

To date, the protective effect of Se against Cd-induced damage has not been studied in a porcine model nor have the effects of multiple Se forms been evaluated in that regard. The objective of the present study was to evaluate the potential effects of a range of Se sources on Cd-induced toxicity in a porcine gut epithelial cell model. Here, IPEC-J2 cells were pre-incubated with each of the Se forms Se-yeast (Sel-plex), selenomethionine (Se-M), sodium selenite (Se-Ni) or sodium selenate (Se-Na) for 48 h, before exposure to $CdCl_2$ for 24 h. The potential cytotoxic effect of $CdCl_2$ was evaluated firstly in the absence of Se, with the nature and extent of the effect of each type of Se form subsequently evaluated relative to this control, using both cell toxicity and DNA damage assays.

2.2) Materials and Methods

2.2.1) Cell culture

IPEC-J2 Porcine jejunal epithial cells (IPEC-J2, DSMZ Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's/Ham's Nutrient Mixture F-12 medium (DMEM/Nutrient Mixture F-12 Ham) supplemented with 10% porcine serum and 1% penicillin-streptomycin at 37^0C in a humidified 5% CO_2 atmosphere (Galaxy S CO2 Incubator, Model No:170-200, RS Biotech Laboratory Equipment Ltd., Irvine, United Kingdom). Cells were passaged just prior to confluence every 3 to 4 days following removal with trypsin/EDTA (all cell culture products were from Sigma Aldrich). All manipulations were done in a Class II biological safety cabinet (BioAire Aura 2000 BS; Bioair Instruments, Pavia, Italy). IPEC-J2 cells are an established non-transformed *in vitro* gastrointestinal model from which results can be extrapolated to the *in vivo* situation (25, 26). Here, IPEC-J2 cells were cultivated in the presence of porcine serum (PS) as opposed to conventional foetal bovine serum FBS. This species-specific growth medium supplementation has been shown to promote the growth of IPEC-J2 which are much

more similar in terms of cell architecture, morphology, transport functions and trans-epithelial resistance to primary pig jejunocytes (27).

2.2.2) Preparation of organoselenium extracts and selenium compounds

Forty milligrams of selenised yeast powder (Sel-Plex®, obtained from Alltech Inc.) was placed in separate 1.5 mL microcentrifuge tubes. Protease enzyme solution [2 mg Protease XIV (Sigma Aldrich) in 0.5 mL of 10 mM Tris-HCl buffer, pH 7.5)] was added and tubes were then vortexed gently for 2 min. Samples were ultrasonicated on ice for 25 s at 80 % amplitude (HTU SONI-130 MiniFIER; G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany). Ice and MilliQ-H_2O were placed into the microwave carousel and the microcentrifuge tubes were placed into the carousel holder. The extraction program was run for 15 min at a power output of 30 W and extracted samples were then centrifuged in a microfuge at 14,000 rpm for 3 min. Supernatants were transferred to 15 mL centrifuge tubes. Pellets were washed with MilliQ-H_2O and vortexed until completely resuspended. Samples were re-centrifuged and the supernatants obtained were pooled with the corresponding first supernatants in the 15 mL centrifuge tube. Pooled samples were made up to 15 mL with MilliQ-H_2O and mixed well. Aliquots (2 mL) were then removed for filtration (0.25 µm) and total organoselenium concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP/MS; Agilent 7700X; Agilent technologies, Maynooth, Ireland), as described previously (8).

Stock solutions of selenomethionine (Se-M), sodium selenite (Se-Ni) and sodium selenate (Se-Na) were prepared in MilliQ-H_2O, diluted to a final Se concentration of 0.4 ppm in cell culture medium and sterilised by filtration through a 0.2 µm PVDF filter. The Se concentrations chosen

for analysis were determined following cell culture toxicity assays (described below) and data is shown in the results section.

2.2.3) Cell culture toxicity assays

Cell Viability was evaluated using the fluorogenic indicator dyes Alamar Blue® (AB) (Bio-Source, Invitrogen) and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester; Molecular Probes, Invitrogen) and a multiwell scanning spectrophotometer (Safire II; Tecan Group Ltd., Männedorf, Switzerland), essentially as described elsewhere (28). A linear relationship between cell number/well and absorption was first established and based on this IPEC-J2 cells were seeded under test conditions at 2 x 10^4 cells/well of a black 96 well flat bottom plate in the presence or absence of the relevant selenocompounds [Se yeast (Se-Y), Se-Na, Se-Ni or Se-M] and were incubated at 37^0C for 48 h. The cells were then insulted with 0.5 ppm, 0.7 ppm or 1 ppm cadmium chloride ($CdCl_2$) and incubated for a further 24 h at 37^oC. Stock solutions (5 mM) of $CdCl_2$ were prepared in MilliQ-H_2O and then diluted in serum free growth medium to achieve the desired final concentrations. In brief, after 24 h of incubation with $CdCl_2$, the medium was removed completely and 100 μL/well of the Alamar-Blue/CFDA-AM working solution was added. Fluorescence was measured 30 min later at 530 nm excitation/590 nm emission for AB and 485 nm/535 nm for CFDA-AM, respectively, using the multiwell scanning spectrophotometer. Average values of triplicates were calculated, and each sample was normalised to its corresponding control.

2.2.4) Comet assay, DNA staining and comet evaluation

Following pre-incubation with Se compounds and subsequent exposure to $CdCl_2$, IPEC-J2 cells were embedded in agarose comet slides (Trevigen, Gaithersburg, USA) at a concentration of 5 x 10^5 cells/mL. Cells were then lysed and the DNA was denatured by treatment with 200 mM NaOH for 30 min. Gel electrophoresis was carried out in an alkaline buffer (200 mM NaOH, 1mM EDTA, pH<13) at 21 volts for 30 min. Gels were then rinsed in MilliQ-H_2O and dehydrated in 70% ethanol.

Cells were stained following electrophoresis with SYBR Gold (Invitrogen, Bioscience Ltd.) for 30 min at the recommended concentration (Trevigen, Gaithersburg, USA). Cells were observed at 10X magnification by fluorescent microscopy at excitation/emission wavelengths of 496 nm/522 nm. Results were obtained by collecting data from at least 50 cells per slide for each control/treatment. Cells were analysed and evaluated based on their comet tail appearance. Quantitative analysis of the comet tails was carried out using OpenComet Image J Software Plug-in (29). Stained cells were also assessed based on previously published grading systems (Grades 0 – 4) with grades 0 and 4 representing those cells with no evident DNA damage and the most DNA damage, respectively (30, 31). After comet analysis, the individual scores for each of the 50 comets were added and an average value for each treatment was generated. Statistical analysis was performed using one-way ANOVA and post hoc Tukey's honest significance test (HSD), statistical significance was set at $P < 0.05$. In addition to quantitative analysis using the scoring system referred to above, the Comet Tail Moment, Olive Tail Moment, % Tail DNA and Tail Length of each of the comets was also determined using the OpenComet software.

2.2.5) TUNEL assay

Apoptosis induced DNA fragmentation was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay which was carried out essentially according to the manufacturer's instructions (Merck Millipore Ireland B.V). Briefly, following Se treatment and incubation with $CdCl_2$, cells were trypsinised and fixed with 4% (w/v) paraformaldehyde in PBS at a concentration of 1 x 10^5 cells/mL and stored in 70% ethanol at -20^{o}C for 18 h prior to staining. Cells were incubated with TDT enzyme and Brd-UTP antibody for 60 min at 37^{o}C followed by rinsing. Anti-BrdU-TRITC staining mixture was added to the cells and incubated for 30 min in the dark. Cells were then centrifuged at 300 x g for 7 min, cell pellets were resuspended in 150µL of rinsing buffer and transferred to a 96 well plate from which data was then acquired on a Guava benchtop Flow Cytometer (Guava easyCyte 8HT; Merck Millipore, Cork, Ireland). All assays were carried out in triplicate and analyses were performed using FlowJo software (FlowJo LLC, Ashland, USA). Each Se treatment was analysed relative to its corresponding control and statistical significance was determined by one-way ANOVA and post hoc Tukey's HSD.

2.3) Results

2.3.1) $CdCl_2$ is toxic to IPEC-J2 cells

The potential cytotoxic effect of $CdCl_2$ on IPEC-J2 cells was analysed over a range of concentrations using an established dual cell viability assay consisting of the fluorogenic indicator dyes Alamar Blue® (AB) and CFDA-AM. AB measures metabolic activity while CFDA-AM reports on membrane integrity. IPEC-J2 cells showed a dose-dependent decrease in cell viability, in both assays, in response to increasing levels of $CdCl_2$ (Fig. 2.1). Two

concentrations of $CdCl_2$ (0.7 ppm and 1 ppm) were chosen for further analysis based on the toxicity curves obtained using both assays.

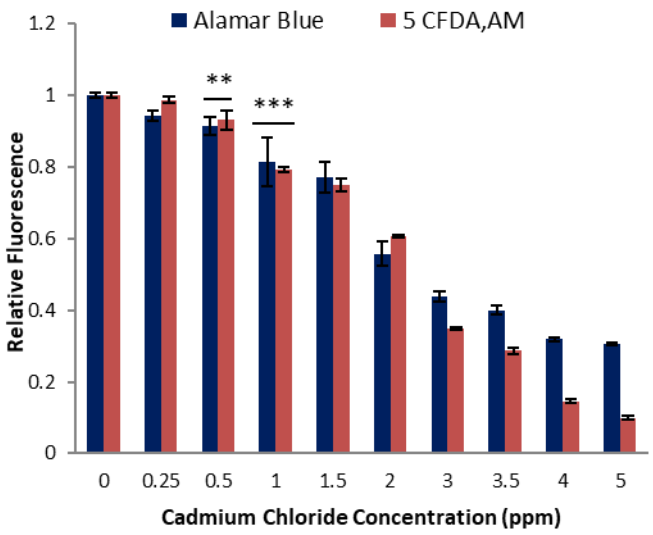


Figure 2.1: Effect on IPEC-J2 cells of exposure to CdCl2 for 24 h as determined using the combined Alamar Blue/CFDA-AM viability assay. Data are expressed as the mean ± SD of triplicate samples and the results for each assay are presented as relative to the corresponding unchallenged control (0 ppm Cd) which was set as 1.

2.3.2) Effect of Se sources on IPEC-J2 viability

The dual viability assay was then used to investigate any potential cytotoxic effects of a range of Se sources on IPEC-J2. The results from both assays showed (Fig. 2.2 a and b) that supplementation of growth medium with Se-Y did not lead to a significant decrease in cell viability at all concentrations tested (up to 1 ppm). Se-Ni had a significant toxic effect ($P<0.001$) however at 0.8 ppm. Se-Na and Se-M had no cytotoxic effect at concentrations between 0.2 ppm – 0.8 ppm, but this was followed by a significant loss in cell viability ($P<0.01$) at concentrations of 1 ppm. Both assays showed the same trend with all Se compounds used. These results concurred with EFSA recommendations for the optimum concentration for Se

supplementation and therefore 0.4 ppm were used in all subsequent experiments designed to investigate the protective effects of Se against Cd-induced DNA damage.

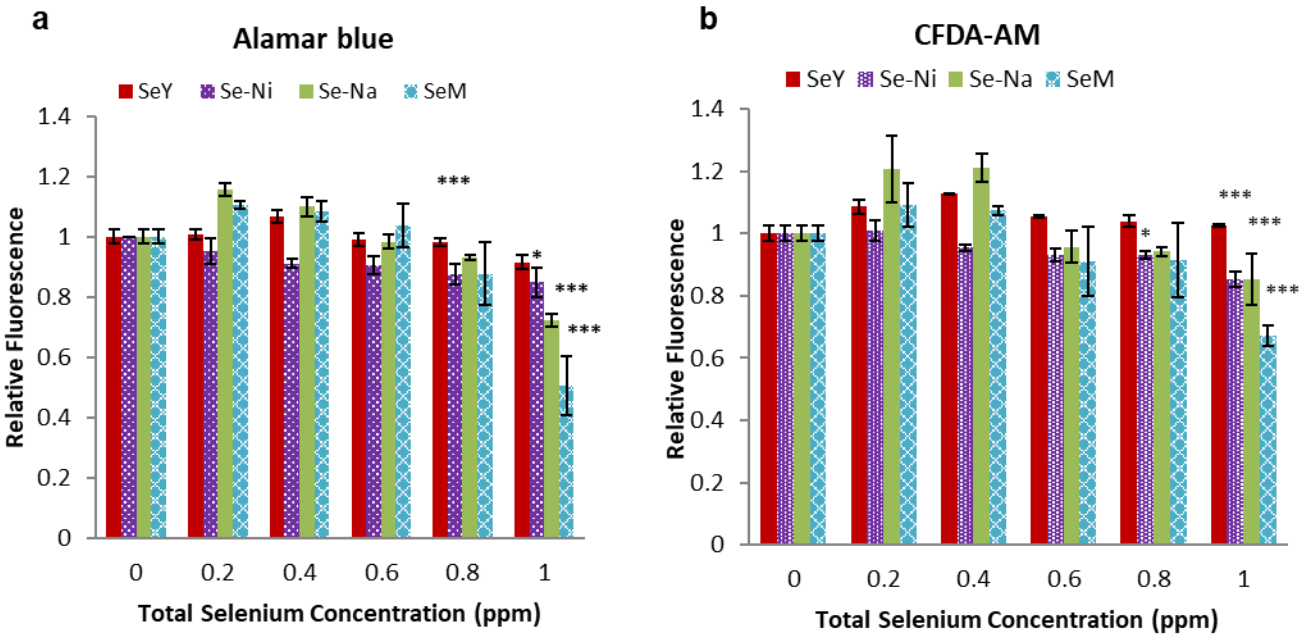


Figure 2.2: Effect on IPEC-J2 cells of pre-incubation with a range of concentrations of Se compounds (Se-Y, Se-Ni, Se-Na and Se-M) for 48 h as determined using (a) AB and (b) CFDA-AM assays. Data are expressed as the mean ± SD of triplicate samples and the results for each assay are presented as relative to the corresponding unchallenged control (0 ppm Se) which was set as 1.

2.3.3) Modulation of Cd-induced cell viability by Se sources

The capacity of various Se sources to protect from Cd-induced cell toxicity was next explored. The results from both assays (Fig. 2.3 a and b) showed that there was a significant protective effect at all Cd concentrations used ($P<0.001$ at 0.5 ppm, $P<0.05$ at 0.7 ppm and $P<0.01$ at 0.5 ppm) when cells were preincubated with Se-Y relative to Cd-insulted cells that had not received Se supplementation. It was also evident (Fig. 2.3) that at all concentrations of Cd, there was significantly greater cell viability when Se-Y was used as supplement when compared to Se-Ni and Se-M. Pre-incubation of IPEC-J2 with Se-Na moderated the decrease in cell viability seen due to 0.7 ppm Cd whereas Se-M pre-incubation reduced the level of cell injury induced

by 1 ppm Cd. In contrast significant decreases in cell viability were observed when Cd treated cells were preincubated with Se-Ni (relative to the no Se control), demonstrating that enhanced Cd-induced cell damage occurred when Se-Ni was used. At all Cd concentrations tested there were significant decreases in cell viability in the Se-Ni treated cells when compared to Se-Y treated cells ($P<0.001$ at 0.7 ppm Cd, $P<0.01$ at 1 ppm Cd; data not shown).

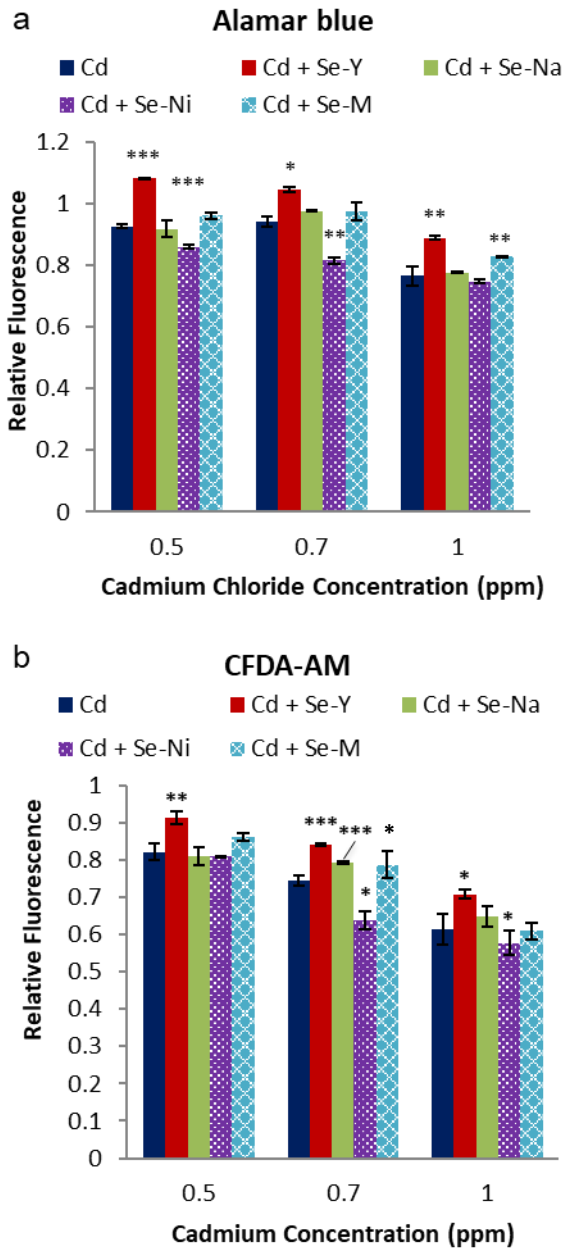


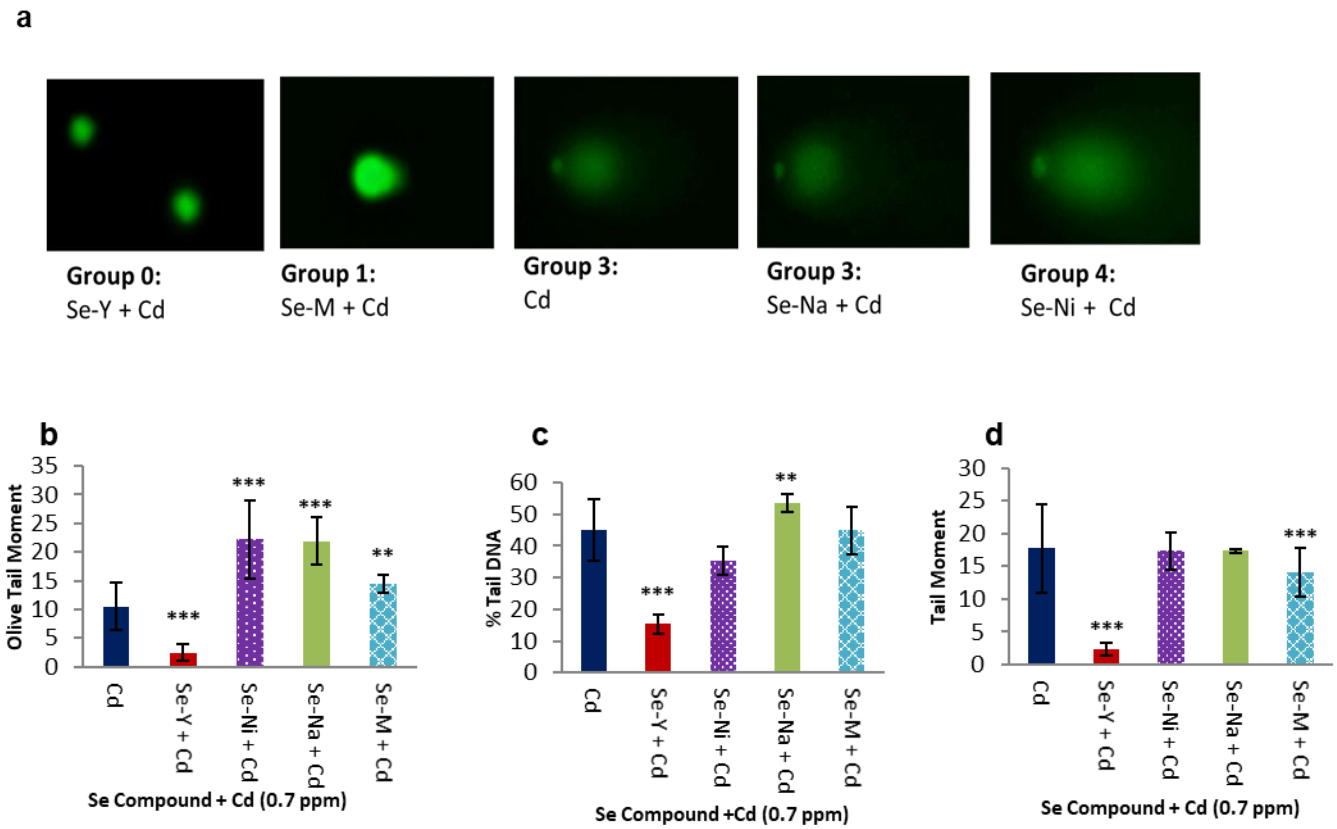
Figure 2.3: Effect on IPEC-J2 cells of pre-incubation with Se compounds (0.4 ppm) for 48 h prior to challenge with $CdCl_2$. (a) AB and (b) CFDA-AM assays. The $CdCl_2$ concentrations used are shown beneath each graph. Data are expressed as the mean ± SD of triplicate samples and the results for each

assay are presented as relative to the corresponding Se-treated unchallenged controls (not shown) which were set as 1. Significant differences were determined by one-way ANOVA and post hoc Tukey's HSD (denoted as *P<0.05, **P<0.01 and ***P<0.001) and highlight comparisons between the data points indicated and the corresponding $CdCl_2$-treated no-Se sample.

2.3.4) DNA damage analysis by Comet assay

The Alkaline Comet assay was employed to assess the extent of Cd-induced single- and double-stranded DNA damage in IPEC-J2 cells which were cultured with or without Se supplementation (Fig. 2.4). It can be seen that there was significantly less Cd-induced DNA damage (0.7 ppm and 1 ppm Cd) when IPEC-J2 were pre-incubated with Se-Y, relative to no Se supplementation, as evident by lower Olive tail moments (Fig. 2.4b and 2.4f), percentage tail DNA (Fig. 2.4c and 2.4g), tail moments (Fig. 2.4d and 2.4h) and tail length values (Fig 2.4e and 2.4i). In contrast, it can be seen from the same figures that significant increases in Cd-induced DNA damage occurred when Se-Ni was used as supplement, as reflected in comet tail lengths and olive tail moments (0.7 ppm Cd), and percent tail DNA and tail moments (1 ppm Cd) when compared to their respective Cd-treated no-Se controls. This indicated that Se-Ni-treated IPEC-J2 were more sensitive to the effects of Cd, as reflected in increased levels of DNA damage. These experiments also showed that although pre-incubation of cells with Se-M followed by Cd insult (0.7 ppm) led in a decrease in tail moment, increases in tail length and olive tail moments were seen when compared to the corresponding Cd treated no-Se control. Significant increases were also noted in three of the four Comet parameters, including tail moment, when the 1 ppm Cd/Se-M combination was used implying that Se-M pretreatment enhanced Cd-induced DNA damage. Pre-incubation of IPEC-J2 with Se-Na resulted in increases to all Comet parameters following exposure to 1 ppm Cd (Fig. 2.4f to 2.4i) relative to Cd-insulted no-Se controls, implying that Se-Na was potentiating the extent of Cd-induced DNA damage at the concentration of Cd used. Overall the evidence suggested that pre-

incubation of IPEC-J2 cells with Se-Y was more effective at protecting cells from Cd-induced DNA damage, at both Cd concentrations used, than supplementation of growth medium with any of the other Se sources used.



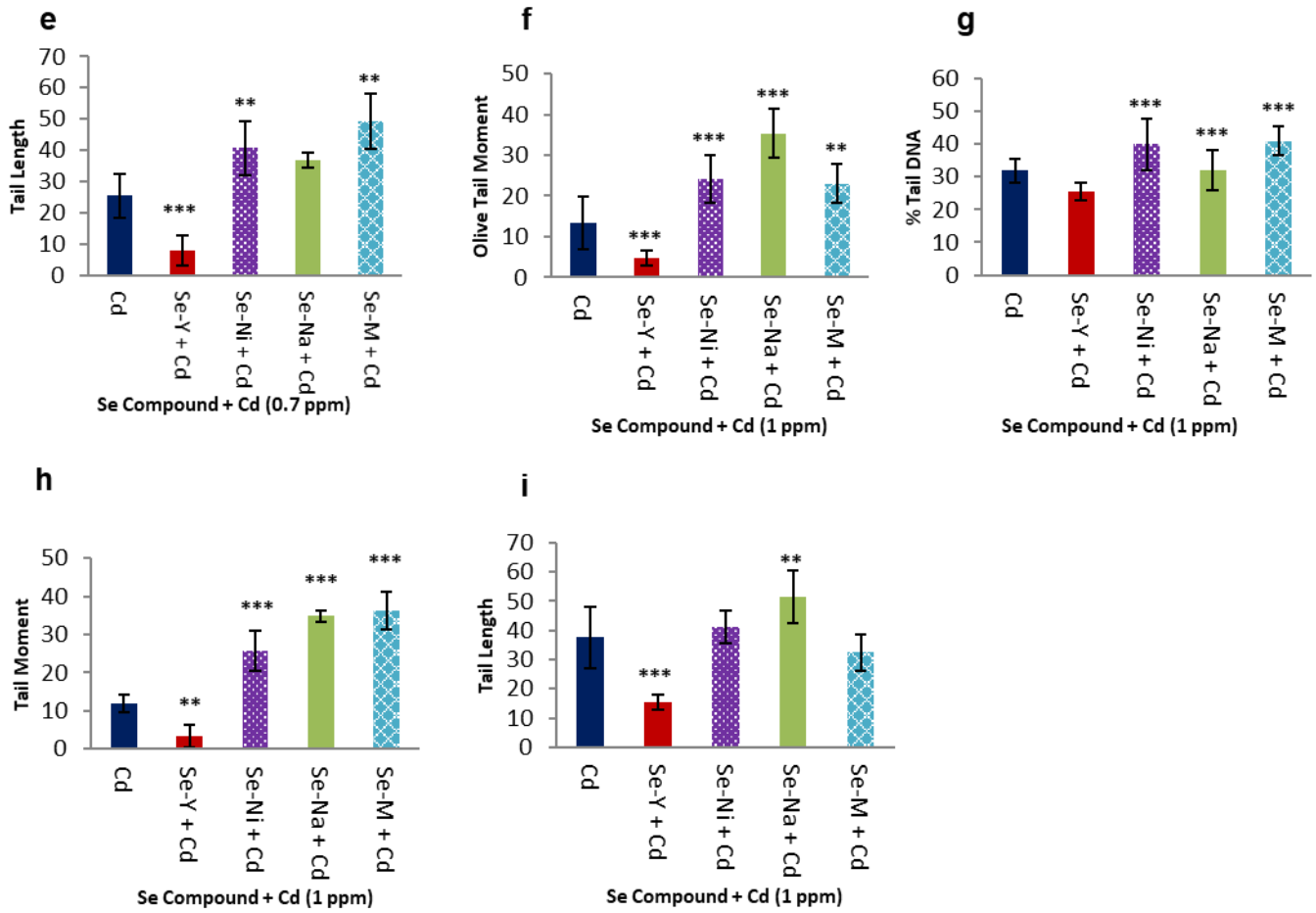


Figure 2.4: Determination of DNA damage in IPEC-J2 cells by Comet assay following challenge with $CdCl_2$. Cells were first pre-incubated for 48 h with various Se sources (0.4 ppm) as indicated underneath each graph, then treated with Cd (0.7 ppm) for a further 24 h prior to analysis by fluorescent microscopy. (a) Representative images of cells that were scored as Group 0 – IV; the corresponding treatments are given underneath. (b) – (i) Comet data from various parameters (indicated on the Y axis of each graph) as determined using OpenComet software. The concentration of $CdCl_2$ used in each case is given underneath each graph. Data expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA and post hoc Tukey's HSD (*P<0.05, **P<0.01 and ***P<0.001) and highlight comparisons between the data points indicated and the corresponding $CdCl_2$-treated no-Se sample.

2.3.5) DNA damage analysis by TUNEL assay

The TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) assay labels cells that contain nicked DNA and is usually deployed to assess DNA fragmentation that is associated with the onset of programmed cell death (apoptosis). Here, TUNEL was used as a means to further investigate the effect of Cd on Se-supplemented IPEC-J2 cells with the degree

of TUNEL-positivity directly reflecting the extent of DNA damage in the cell population under analysis. A significant increase in the TUNEL-positive (apoptotic) cell population was seen following treatment with $CdCl_2$ (0.7 ppm and 1 ppm) indicating the induction of DNA damage and the onset of DNA fragmentaton (Fig. 2.5a and 2.5b). Pre-incubation of cells with Se-Y was seen to significantly inhibit the extent of Cd-induced TUNEL-labeling at both Cd concentrations used as reflected in correspondingly increased TUNEL-negative populations. Similarly, pre-incubation with Se-M followed by exposure to Cd correlated with a significant increase in the non-apoptotic population, although not to the same extent as Se-Y when 0.7 ppm Cd was used as insult. In contrast, prior supplementation of cells with either Se-Na or Se-Ni had no protective effect with either concentration of Cd. Overall this data showed that pre-incubation with either Se-Y or Se-M protected IPEC-J2 cells from Cd-induced DNA fragmentation and apoptosis whereas the inorganic Se compounds used did not.

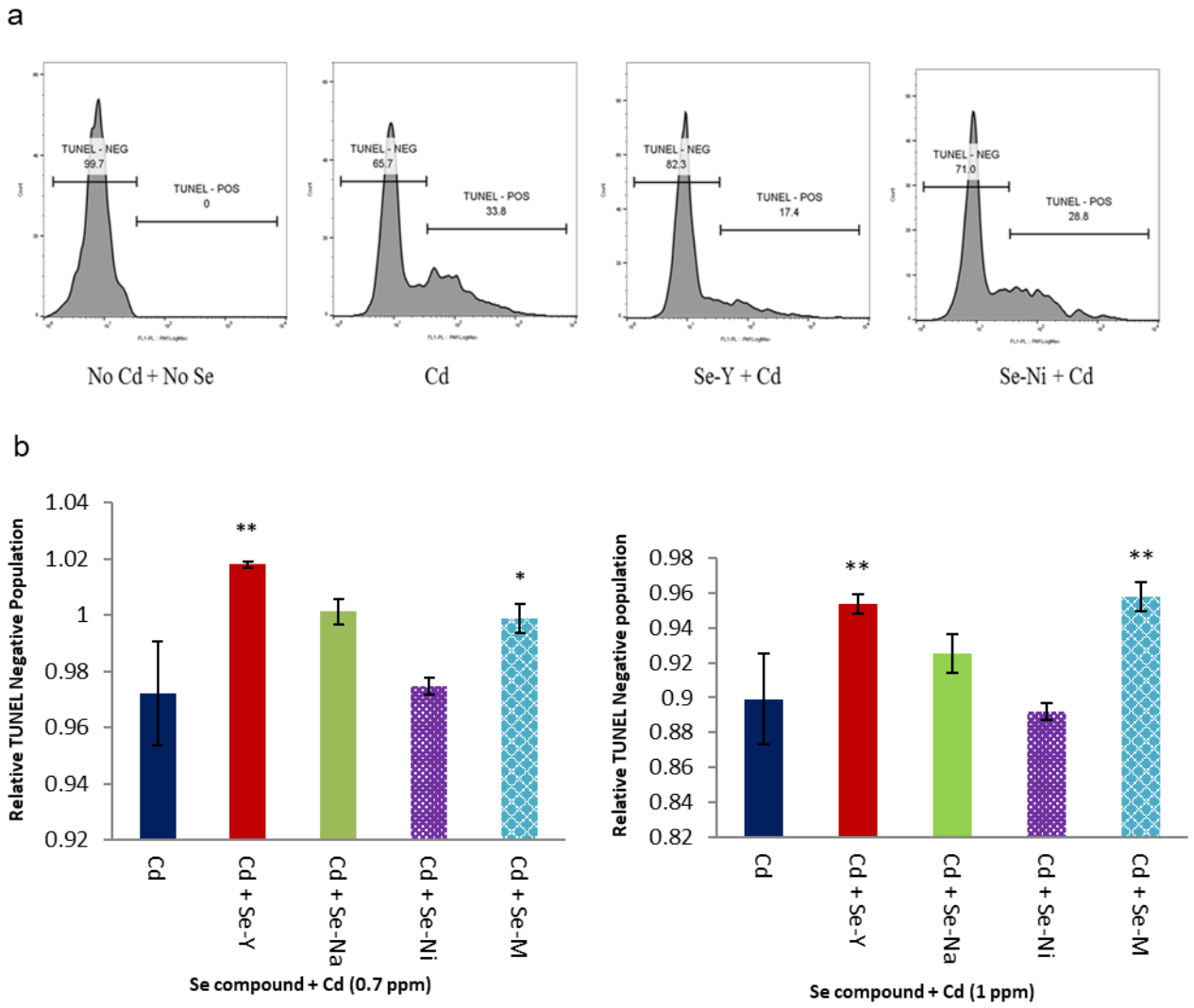


Figure 2.5: TUNEL analysis of IPEC-J2 cells following 48 h pre-incubation with Se sources (0.4 ppm) and subsequent exposure to $CdCl_2$ for 24 h. (a) Representative histogram plots of IPEC-J2 cells treated as described underneath each image. TUNEL-negative and TUNEL-positive populations are highlighted in each panel by bars (left and right-hand sides, respectively). (b and c) DNA damage was induced in IPEC-J2 using $CdCl_2$ at 0.7 ppm (b) and 1 ppm (c) and TUNEL-Negative populations are presented as a fraction of the same group in the corresponding $CdCl_2$- treated/no Se sample. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA and post hoc Tukey's HSD (***P<0.001) and highlight comparisons made with the corresponding Cd-treated no-Se sample.

2.4) Discussion

IPEC-J2 is increasingly being used as a superior alternative to transformed cell lines, such as Caco-2, for gastrointestinal studies including probiotic screening, feed additive screening and immune and inflammatory studies. It was reasoned that the use of PS (as opposed to FBS) as a

growth medium supplement would further enhance the quality of this model system (27) and that the AB/CFDA-AM dual assay, which measures both mitochondrial enzyme activity and membrane integrity, would be a robust assay combination for assessing cytotoxicity effects. The study presented here represents the first report involving $CdCl_2$ and IPEC-J2. The Cd toxicity data generated here (Fig. 2.1) concurs with that derived elsewhere with a range of cells lines including HepG2, 1321N1 and HEK 293 using MTT and LDH assays. Measurable decreases in cell viability were observed after 24 h with 0.25 ppm $CdCl_2$ and a significant decrease was seen in all cell lines with 2.5 ppm $CdCl_2$ (32, 33). Another report investigating Cd-induced damage on LLC-PK_1 cells showed that 1ppm $CdCl_2$ induced apoptosis after 18 h and therefore selected $CdCl_2$ concentrations of 0.5ppm and 1ppm for their study (32). It would appear therefore that Cd-induced cell damage in the porcine model developed here is comparable in terms of cell viability to that observed for multiple human cell lines.

Although Se has been reported to have cytotoxic properties and to prevent oxidative stress *in vitro*, at higher concentrations Se can become pro-oxidant and lead to free oxygen radical production and the generation of oxidative stress. It was important therefore to determine the concentration range in which Se supplementation resulted in cytotoxicity. The toxic effects of Se are known to be concentration and composition dependent (34) and both inorganic and organic forms of Se can exhibit pro-oxidant effects leading to the induction of cell apoptosis at high concentrations (35). EFSA Food and Feed regulations requires that total Se in animal feed products does not exceed 0.2 ppm to 0.4 ppm (36). In the present study, Se compounds were assayed over a concentration range of 0.2 ppm to 1 ppm (Fig. 2.2). Se-Y retained an ameliorative effect at all concentrations tested whereas cytotoxicity was detectable for both Se-M and Se-Na at higher concentrations. Se-Ni was seen to promote a modest decrease in cell viability starting as low as 0.4 ppm. Indeed, Se-Ni has been reported elsewhere to promote damage to HepG2 cells after 24 h over a concentration range of 0.25 to 1.25 ppm (37), and to

induce cytotoxicity when administered at a concentration of 1 mg/kg in rodent (17) and chicken studies (16). Importantly the choice of 0.4 ppm in the present study concurred with EFSA guidelines for the optimum concentration for Se supplementation. The results obtained here clearly demonstrated that Se-Y did not exhibit any cytotoxic effects at any of the concentrations analysed, and that this was the only form of Se for which this effect was observed. In the porcine model therefore, it is clear that Se-Y is the safest form of supplementation, up to the maximum concentration analysed, namely 1 ppm.

It was shown that Se-Y was significantly more effective at preventing a decrease in cell viability due to Cd exposure than inorganic Se sources (Fig. 2.3). That an organic Se source affords more protection correlates with general observations that inorganic Se is generally not as effective at protecting cells from oxidative stress. This is likely due to the difference in metabolism, absorption and retainment of organic versus inorganic Se compounds (9). In human cell lines, it was observed that Se-Na was more effective than Se-Ni at promoting resistance to Cd insult. This difference between these inorganic Se sources has been noted elsewhere using the melanoma cell line (HTB 140), human melanocytes and keratinocytes (38). In one study a range of cell lines was shown to be more sensitive to treatment with Se-Ni as opposed to Se-Na, as evidenced by decreased cell growth (38). Another study investigating the effects of Se-M on LNCaP prostate cancer cells reported that Se-M had a significant protective effect in response to oxidative stress (15). While these studies looked at differences either between inorganic Se sources or explored the effect of organic Se sources, the effects of Se-Y, Se-M, Se-Ni and Se-Na on Cd-induced damage have not been directly compared in a single study to date. Animal studies have, however, illustrated that organoselenium is more bioavailable and more readily incorporated into biomass than are inorganic Se sources (13). In the present study, it was clearly demonstrated that organic Se sources inhibited Cd-mediated reductions in cell viability, while inorganic sources promoted these reductions, with Se-Y

demonstrating the greatest ameliorative effect in that regard and also being the only Se source that did not lead to decreased cell viability when used alone (in the absence of Cd) as supplement.

The Alkaline Comet assay detects and quantifies both single stranded and double stranded DNA breakage. Using this technique cells are lysed *in situ* in agarose, electrophoresed and stained with a DNA-binding dye. Following electrophoresis, fragmented DNA migrates out of the nucleoid towards the anode forming a comet shape while undamaged DNA migrates more slowly under the influence of the electric field (39). The data derived in the present study using the Comet assay shows that pre-incubation of IPEC-J2 cells with Se-Y afforded significant protection against Cd-induced DNA damage (Fig. 2.4). The Comet assay results therefore correlate directly with the cell viability results obtained. Se-Y exhibited the greatest ameliorative effect against Cd-induced DNA damage, followed by Se-M, with the inorganic Se sources in fact enhancing the extent of damage due to Cd. Elsewhere, the Comet assay was used to show a comparable effect on lead-induced DNA damage to HepG2 cells following Se pre-treatment, where Se-Y lead to a decrease in lead-induced DNA damage (8). The same study also showed that Se-Ni pre-treatment resulted in a strong genotoxic effect and a significant increase in DNA strand breakage. The differing effects of Se-Ni and Se-Na supplementation (Fig. 2.4) is also in agreement with other studies in which the Comet assay was also used to demonstrate enhanced DNA damage promoted by Se-Ni relative to Se-Na in HepG2 and melanoma cells (38)(37).

In the porcine model investigated here, greater levels of DNA damage were noted in Se-M treated IPEC-J2 cells, relative to those treated with Se-Y. Interestingly, a study in which the digestion and oxidation of different Se compounds was analysed, concluded that Se-M concentrations decreased in the small intestine concomitant with the appearance of the oxidation product SeMetO, suggesting that Se-M was prone to targeting by ROS (40). It has

also been reported that the form of Se-M which is used as an additive in animal feed is a synthetic form of L-Se-M. The latter contains D-Se-M as an impurity which is not metabolised efficiently and can build up in organs and tissues leading to toxic effects in the body (41). In contrast, the Se in Se-Y is highly bioavailable, bioactive and easily absorbed into the bloodstream (42). Another study which investigated differing gene profiles in response to Se-Y and Se-M using a mouse model, suggests that Se-Y comprises several different protein-bound Se compounds in addition to selenomethionine (42). This indicates that not only is the chemical form of Se important for determining its ameliorative effect, but also the nature of protein-bound Se complexes. It may therefore be the case that protein-bound forms of Se which lead to increased bioavailability of Se result in a greater protective effect. However, further studies are necessary to explore this possibility.

Here, the TUNEL assay was also used to show that pre-incubation of IPEC-J2 cells inhibited Cd-induced apoptosis-associated DNA fragmentation (Fig. 2.5). Furthermore, it was evident from the same data that neither Se-Na nor Se-Ni provided any protective effect. These results corroborated the Comet assay data shown above. Elsewhere studies using HepG2 and leukemic HL60 cells showed substantial increases in TUNEL-positive populations following treatment with Se-Ni (37), (43). It was also observed that pre-incubation of IPEC-J2 with Se-M coincided with a significant decrease in Cd-induced TUNEL-positive cell numbers relative to no-Se controls, although the effect was not as significant as that observed following Se-Y pretreatment when 0.7 ppm Cd was used. The latter point was not supported from the corresponding Comet assay data however. The fact that Cd-induced apoptosis begins with DNA strand breakage and eventually leads to apoptosis (44), offers a potential explanation for observed higher levels of DNA damage and lower rates of apoptosis as determined by Comet and TUNEL assays, respectively.

In summary, the effects of multiple forms of Se supplementation on cell viability and DNA damage in IPEC-J2 cells following Cd exposure were evaluated. Overall the data showed that Se protective effects are both composition- and dose-dependent as evident from a range of cell viability and DNA damage assays. It was demonstrated that organic forms of Se exhibited lower levels of cytotoxicity and genotoxicity than inorganic Se forms in this porcine gut epithelial model. At all concentrations analysed, Se-Y did not exhibit any cytotoxic effects and it is postulated that this may be as a result of the nature of the protein-bound Se complexes. It was also demonstrated that organic Se species, when used at EFSA guideline levels as a growth supplement prior to Cd exposure, have an ameliorative effect against Cd-induced DNA damage in the IPEC-J2 model system whereas inorganic Se sources do not, and can in fact enhance the negative effects of Cd-induced damage. These results are relevant to the Agri-Food industry and highlight the negative implications of supplementation with inorganic Se forms, as well as the potential for nutritional supplementation in the form of Se-Y to protect gut integrity from damage caused by the environmental contaminant Cd.

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Chapter Three: Selenised yeast sources differ in their capacity to protect porcine jejunal epithelial cells from cadmium-induced toxicity and oxidised DNA damage.

Abstract

In recent years there has been increasing interest in the use of selenised yeast (Se-Y) as an antioxidant feed supplement. Here, three selenised yeast products are differentiated in terms of bioefficiency and the ameliorative effect on Cadmium (Cd) toxicity in porcine epithelial cells. A porcine digestion *in vitro* model was chosen to more accurately simulate the bioavailability of different Se-Y preparations, allowing a comprehensive understanding of the bio efficiency of each Se-Y compound in the porcine model. To elucidate a possible mechanism of action of selenium a number of bioassays were applied. Levels of Se dependent antioxidant enzymes (glutathione peroxidase and thioredoxin reductase) were evaluated to analyze the ROS neutralizing capacity of each Se-Y compound. The effects of Se-Y sources on Cd-induced DNA damage and apoptosis-associated DNA fragmentation was assessed using comet and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, respectively. Lesion-specific DNA damage analysis and *in vitro* DNA repair assay determined the DNA repair capacity of each Se-Y source. The results presented in this study confirm that the ability of different commercially available Se-Y preparations to enhance a range of cellular mechanisms that protect porcine gut epithelial cells from Cd-induced damage is concentration-dependent and illustrates the difference in bioefficiency of different Se-Y compounds.

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3.1) Introduction

Selenium (Se) is an essential trace element which is crucial for human and animal health. Se has been shown to play important roles in many physiological functions including antioxidant function, reproduction, immune responses, DNA synthesis and thyroid hormone production (1). Se is commonly consumed as a nutritional supplement and is frequently utilised as an additive in animal feed products. Se can occur as either inorganic Se including selenites (Se-Ni) and selenates (Se-Na) or organic Se including selenomethionine (Se-M) or selenized yeast (Se-Y) (2). Inorganic Se is the form most commonly used for Se supplementation in animal feed products. It has been reported however that microorganisms present in rumen of animals such as sheep and pigs may have a negative effect on Se bioavailability by reducing inorganic Se into inorganic selenide which cannot be absorbed leading to negative economic and environmental consequences due to unabsorbed Se being excreted by the faecal route (3).

In recent years, there has been increased interest in the use of Se enriched yeast (Se-Y) preparations in animal feed products, due to the demonstrated superior absorption of organic Se forms relative to inorganic Se (4) and the increasing evidence illustrating that organic Se forms offer an enhanced amelioration of oxidised damage (5,6). Live yeast cells can absorb Se-Ni in the surrounding culture medium and convert inorganic Se into Se-M resulting in a selenized yeast preparation. Studies examining the Se binding efficiency of different yeast strains have focused mainly on the Se tolerant *Saccharomyces cerevisiae* and *Candida utilis* (also known as Torula yeast) (7, 8). To date, only Se tolerant strains of *S. cerevisiae* and *C. utilis* have been used by commercial manufacturers for Se-Y production as they have been granted GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (FDA) (9) and are approved for animal and human use by the European Food Safety Authority (EFSA; EFSA regulations specify that the total Se concentration in animal feed must not

exceed 0.4 ppm and that supplementation with exogenous Se must not exceed 0.2 ppm) (EFSA 2011; EFSA 2013). The level of Se bound in the organic form in Se-Y preparations can reach 3000 ppm and is influenced by the yeast strain, growth conditions and initial inorganic Se concentration (12). Nutritional Se-Y preparations contain varying amounts of free Se-M, peptide bound Se-M and total Se-M along with different protein and peptide compositions (13). Although the main Se component of Se-Y is Se-M, it has been observed that gene profiles differ in response to Se-Y and Se-M supplementation in a mouse model, suggesting that Se-Y comprises several different protein-bound Se compounds in addition to Se-M (14). It has also been reported that the form of Se-M which is used as an additive in animal feed is a synthetic form of L-Se-M. The latter contains D-Se-M as an impurity which is not metabolised efficiently and can build up in organs and tissues leading to toxic effects in the body (15). A recent study in which the digestion and oxidation of different Se compounds was analysed, concluded that Se-M concentrations decreased in the small intestine coupled with the appearance of the oxidation product selenomethionine selenoxide (SeMetO) (16). Se-Y is an appealing Se source for nutritional supplementation due to its activity as a selenoprotein precursor and its low toxicity. In addition, there is evidence that Se-Y is highly bioavailable, bioactive and has the ability to increase selenoenzyme activity (5, 17). Se-Y has also been reported to be more bioavailable than inorganic Se sources and considerably less toxic at supranutritional levels than Se-Ni and Se-Na (2, 3). Recent studies have provided evidence that supplementation with organic Se affords protection against oxidised DNA damage (6, 18–20).

It is widely recognised that the concentration of Se impacts its biological activity, and increasingly acknowledged that the chemical form of Se plays a key role, with organic forms of Se demonstrating a positive impact and inorganic forms resulting in toxic effects (13, 20). Regarding organic forms of Se, it is increasingly apparent that not all selenised yeasts (Se-Y)

are comparable, but more appropriately may be viewed as discrete products/complexes (22). To date, over 60 Se-containing species have been detected in Se-Y (23), with significant differences detected between Se-Y products in terms of Se-M content, Se containing protein abundance and associated metabolic pathways (13). The uniqueness of these organic Se compounds are attributed to the differential deposition of selenium into the numerous peptides and proteins that are present within individual yeast fractions. To date, the differences between these yeasts have been explored primarily in terms of differences in Se bioavailability (22, 24). Given the wide array of Se species which can exist within these yeasts, the possibility exists that not only overall Se bioavailability but also the mode of action of bioavailable Se could be impacted by the particular traits of the individual yeast strains.

In this study, the biological impact of different Se-Y preparations was assessed. Specifically, the effects of different Se-Y formulations on cadmium-induced toxicity in a porcine gut epithelial cell model were evaluated. Cadmium (Cd) is a bio-toxic heavy metal of environmental concern. Following cellular uptake, Cd displaces Fe^{2+} and Cu^{+} ions which in turn induce the production of Reactive Oxygen Species (ROS), of which the gastrointestinal tract is a primary target (25, 26). Using Cd-insulted porcine jejunal epithelial cells (IPEC-J2) cultivated *in vitro*, the data showed that Se-Y preparations from three different sources varied considerably in their capacity to maintain cell viability, to prevent oxidised DNA damage, to promote anti-oxidant enzyme activity and to modulate the cellular capacity for DNA repair. It is concluded that the antioxidant effects of Se-Y preparations, when used at European Food Safety Authority (EFSA) guideline levels, are both source/composition and dose-dependent.

3.2) Materials and Methods

3.2.1) Organoselenium extract preparation and selenium compounds

Organoselenium extracts were prepared from three different commercially available sources of selenised yeast (Se-YA, Se-YB and Se-YC). The yeast products were sourced from different manufacturers who generate dried and inactivated preparations from different strains of *Saccharomyces cerevisiae* (13). Extracts were prepared using a porcine digestion model (27) and experimental volumes outlined elsewhere (28) as follows: 500 mg of each yeast powder was placed in separate 50 mL polystyrene tubes. Five millilitres of sodium phosphate buffer (0.1 M, pH 6) was added to each along with 2 mL of 0.2 M HCl and the pH was adjusted to 2.0. Pepsin (Sigma Aldrich) and chloramphenicol (Sigma Aldrich) were then added to final concentrations of 6 Units/mL and 0.3 mg/mL, respectively. Digestions were incubated for 2 h with gentle shaking at 39^0C. Two mL of sodium phosphate buffer (0.2 M, pH 6.8) and 1 mL of 0.6 M NaOH were then added to each tube and the pH was adjusted to 6.8. Pancreatin solution (Sigma Aldrich) was added to a final concentration of 3mg/mL and the tubes were then incubated for 4 h with gentle shaking at 39^0C. Tubes were then centrifuged at 16,000 x g for 15 min at 4^0C. The supernatants obtained were transferred to ultrafiltration tubes with a molecular weight cut-off of 10,000 Daltons (Sartorius, Gottingen, Germany) and these were centrifuged at 8,000 x g for 3 h at 4^0C. Following centrifugation, the filtrates obtained were aliquoted into microfuge tubes and stored at -70^0C until use. Total organoselenium concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent Technologies, Maynooth, Ireland). All Se-Y preparations were aseptically filtered (0.25 μm) and diluted in DMEM/Nutrient Mixture F-12 Ham to final Se concentrations of either 0.2 ppm or 0.4 ppm immediately prior to use in cell culture. Solutions of Se-M and Se-Ni were prepared in MilliQ-H_2O and processed using the porcine digestion model as described above.

3.2.2) Cell culture and cell toxicity assays

IPEC-J2 Porcine jejunal epithelial cells (IPEC-J2, DSMZ Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's/Ham's Nutrient Mixture F-12 medium (DMEM/Nutrient Mixture F-12 Ham) supplemented with 10% porcine serum and 1% penicillin-streptomycin at 37^0C in a humidified 5% CO_2 atmosphere (Galaxy S CO2 Incubator, Model No:170-200, RS Biotech Laboratory Equipment Ltd., Irvine, United Kingdom). Cells were passaged just prior to confluence every 3 to 4 days following removal with trypsin/EDTA (all cell culture products were from Sigma Aldrich). All manipulations were done in a Class II biological safety cabinet (BioAire Aura 2000 BS; Bioair Instruments, Pavia, Italy). IPEC-J2 cells are an established non-transformed *in vitro* gastrointestinal model from which results can be extrapolated to the *in vivo* situation (29, 30). Here, IPEC-J2 cells were cultivated in the presence of porcine serum (PS) as opposed to conventional foetal bovine serum FBS. This species-specific growth medium supplementation has been shown to promote the growth of IPEC-J2 which are much more similar in terms of cell architecture, morphology, transport functions and trans-epithelial resistance to primary pig jejunocytes (31). Cells were incubated in the presence or absence of the individual Se sources and incubated at 37^0C for 48 h. They were then insulted with 0.5 ppm, 0.7 ppm and 1 ppm cadmium chloride ($CdCl_2$) and incubated for a further 24 h at 37^oC. Stock solutions (5 mM) of $CdCl_2$ were prepared in MilliQ-H_2O and then diluted in serum free growth medium to achieve these desired final concentrations. Cell viability was evaluated using the fluorogenic indicator dyes Alamar Blue® (AB) (Bio-Source, Invitrogen) and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester; Molecular Probes, Invitrogen) and a multiwell scanning spectrophotometer (Safire II; Tecan Group Ltd., Männedorf, Switzerland), as described previously (20).

3.2.3) Comet and TUNEL assays

The Alkaline comet assay was employed to assess the extent of DNA damage in IPEC-J2 cells following pre-incubation with Se sources and subsequent exposure to $CdCl_2$ (32). Cells thus treated were embedded in 0.7% agarose on comet slides (Trevigen, Gaithersburg, USA) at a concentration of 5 x 10^5 cells/mL and processed as described previously (20). In summary, cells were lysed for 18 h, immersed in alkaline unwinding solution and electrophoresed at 1V/cm for 30 min. At least 50 cells were evaluated per slide for each control/treatment. Quantitative analysis of comet tails was carried out using OpenComet Image J Software Plug-in (31-33). The individual scores for each of the 50 comets were added and a mean value for each treatment was generated. In addition to quantitative analysis, % Tail DNA of each of the comets was also determined using the OpenComet software.

Apoptosis induced DNA fragmentation was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Merck Millipore Ireland B.V) according to the manufacturer's instructions. Data was acquired on a Guava benchtop flow cytometer (Guava easyCyte 8HT; Merck Millipore, Cork, Ireland). In all cases triplicate samples from individual experiments were prepared and analyses were performed using FlowJo software (FlowJo LLC, Ashland, USA).

3.2.4) Lesion-specific DNA damage analysis

Cells were analysed for lesion-specific DNA damage following Se/$CdCl_2$ treatment regimens using a modified comet assay which included individual DNA repair enzymes (32, 34). Thus, the enzymes (i) *E. coli* Endouclease III (Endo III or also known as Thymine Glycol-DNA Glycosylase; Trevigen, 4045-01K-FM) and (ii) FPG (formamidopyrimidine-DNA glycosylase; also known as 8-oxoguanine DNA glycosylase; Trevigen 4040-100-FM) were

used according to the manufacturer's instructions, with particular attention paid to minimising endogenous DNA damage and repair occurring during sample preparation. The optimal concentrations of Endo III and FPG to use (4.5 U/mL and 3.75 U/mL respectively) were first established following titration experiments using IPEC-J2 cells that had been treated with Cd at 0.7 ppm for 24 h. Buffer-only control samples were also run in parallel whereby net enzyme sensitive sites could then be determined. Results were expressed as % DNA in tail.

3.2.5) Glutathione peroxidase and thioredoxin reductase assays

Glutathione peroxidase (GPx) and thioredoxin reductase (TRx) activities were determined using a GPx Assay kit (Cayman Chemicals, Item No. 703102) and a TRx Assay kit (Sigma Aldrich, CS0170), respectively, as per manufacturer's instructions. IPEC-J2 cells were lysed in the appropriate buffers, harvested using a cell scraper and lysates were stored at -80^{o}C until use. The protein concentrations of all cell lysates were determined by Bradford assay (Sigma Aldrich, B6916).

3.2.6) Modified Comet *in vitro* DNA repair assay

There were three stages to the modified comet *in vitro* DNA repair assay, namely; substrate cell preparation, DNA repair extract preparation and the modified comet-DNA *in vitro* repair assay itself. (i) Substrate cells were prepared essentially as described elsewhere (32, 35, 36) with the following modifications: IPEC-J2 were cultivated in DMEM medium, treated with/without 0.7 ppm $CdCl_2$ for 24 h by which time they were nearing confluence. Cells were then collected by trypsinisation, placed on ice and a sample was counted. They were then washed with phosphate buffered saline (PBS) and centrifuged at 700 x g at 4^{o}C for 5 min. Cells were resuspended at ~1-2 x 10^6 /mL and frozen in 0.5 mL aliquots at -80^{o}C using a Nalgene freezing container and stored at -80^{0}C until required. (ii) Cellular 'DNA repair extracts' were prepared as follows: IPEC-J2 cells were cultivated in the presence and absence of Se sources

for 48 h, harvested and collected by centrifugation as described above. Cell pellets were resuspended in ice-cold freezing medium at ~5 x 10^6/mL, frozen and stored at -80ºC until processed further. Cells were thawed and centrifuged at 700 x g for 5 min at 4ºC, washed with PBS and re-centrifuged. Cell pellets were then resuspended in 3x extraction buffer (10x buffer: 45 mM HEPES; 0.4 M KCl; 1 mM EDTA; 0.1 mM dithiothreitol; 10% (v/v) glycerol; adjusted to pH 7.8 with KOH) at 5 x 10^6/mL and centrifuged at 2000 x g for 5 min at 4ºC. Supernatants were discarded and pellets were resuspended in 50 µL extraction buffer and 15 µL of 1% Triton X-100, vortexed briefly and incubated on ice for 10 min. Tubes were then centrifuged at 15,000 x g for 5 min at 4ºC and supernatants were collected into new microfuge tubes. The protein concentration of each extract preparation was determined using the Bradford Assay (Sigma Aldrich) as per manufacturer's instructions. (iii) The modified comet-DNA repair assay was carried out as follows: $CdCl_2$-exposed and non-exposed substrate cells were thawed, washed with cold PBS and centrifuged at 700 x g for 5 min. Supernatants were removed and a volume of low melting agarose was added to reach a final concentration of 2 x 10^5/mL, after which 70 µL of each suspension was spread evenly onto a CometSlide™ (Trevigen, 4250-004-03). Slides were incubated at 4ºC for 30 min and then submerged in Comet Lysis Buffer (Trevigen, 4250-010-01) for 18 h. Slides were washed three times for 5 min each in BER buffer (40 mM HEPES, 0.5 mM EDTA, 0.2 mg/mL BSA, 0.1 M KCl, adjusted to pH 8 with KOH). Slides were then placed on a metal plate on ice and 4 volumes of BER buffer was added to each extract preparation. Control solutions were prepared using buffer A containing 0.25% Triton X-100 and buffer B in a 1:4 ratio. Fifty microliters of diluted DNA repair extract or control solution was then added to each gel prior to incubation of gels at 37ºC in a humidity chamber for 30 to 60 min. Slides were then removed, placed briefly on ice followed by immersion in electrophoresis buffer for 30 min prior to electrophoresis (1V/cm for 30 min). After electrophoresis, gels were neutralized in cold PBS for 10 min and left to dry overnight. Slides

were then stained using SYBR Gold (Invitrogen; 1:10,000 dilution) for 30 min, washed twice with dH_20 for 5 min each, allowed to dry completely and then analysed by fluorescent microscopy. IPEC-J2 cells were incubated with reaction buffers containing FPG and Endo III enzymes in the absence of Cd as a negative control, and Cd damaged IPEC-J2 cells were incubated with reaction buffers containing FPG and Endo III enzymes as positive control. A background control where IPEC-J2 cells were incubated with reaction buffers in the absence of enzymes and Cd, a treatment control where Cd damaged IPEC-J2 cells were incubated with reaction buffers in the absence of enzymes, and a specificity control where IPEC-J2 cells were incubated with Se-IPEC-J2 cell extract in the absence of Cd were also carried out. Repair-related DNA incisions were calculated using the formula:

Net DNA repair activity = (Cd-induced DNA incisions $_{\text{Se exposed cell extract/buffer incubation}}$)

- (undamaged cell DNA incisions $_{\text{Se exposed cell extract/buffer incubation}}$)

- (Cd-induced DNA incisions $_{\text{no Se exposure cell extract/buffer incubation}}$).

3.2.7) Statistical Analysis

The results of this study were analysed by one-way analysis of variance (ANOVA) and using post hoc Tukey's honest significance test (HSD). $P<0.05$, $P<0.01$ and $P<0.001$ were considered statistically significant. Data presented in this study are shown as mean values of triplicate samples and the standard deviation (SD) within triplicate samples is represented on the graphs. Each Se treatment was analysed relative to its corresponding control.

3.3) Results

3.3.1) Se-Y sources differ in their capacity to protect IPEC-J2 cells from Cd-induced cell toxicity

The potential of different Se-Y sources to protect IPEC-J2 cells from Cd-induced cell toxicity was investigated using a dual cell viability assay consisting of the fluorogenic indicator dyes Alamar Blue® (AB) and CFDA-AM. AB measures metabolic activity while CFDA-AM reports on membrane integrity. In general data from both assays agreed, showing that supplementation of growth medium with individual Se-Y compounds prior to insult with Cd at 0.5 ppm resulted in a preservation or modest increase in IPEC-J2 viability relative to Cd-insulted non-Se supplemented control cells (Fig. 3.1 A – D). The ameliorative effects of Se-Y were more pronounced at the higher concentration of Se used (0.4 ppm). Significant differences between Se-Y sources were evident at higher Cd concentrations (0.7 and 1 ppm) whereby supplementation with Se-YB and Se-YC at 0.4 ppm either failed to improve or in fact enhanced the negative effects of 0.7 ppm and 1 ppm Cd exposure. In contrast supplementation with Se-YA correlated with increased cell viability, relative to unsupplemented controls, at all concentrations of Cd tested, showing that Se-YA supplementation was more effective at preventing Cd-induced cell injury than Se-YB or Se-YC. At 0.2 ppm, Se-YA maintained cell viability levels during Cd challenge at 0.7 ppm whereas the other Se sources either did not protect (Se-YC and Se-Ni) or else enhanced the negative effects of Cd (Se-YB and Se-M). In the absence of $CdCl_2$, supplementation with Se-Y sources did not lead to a significant decrease in cell viability at all concentrations tested (up to 1 ppm; data not shown).

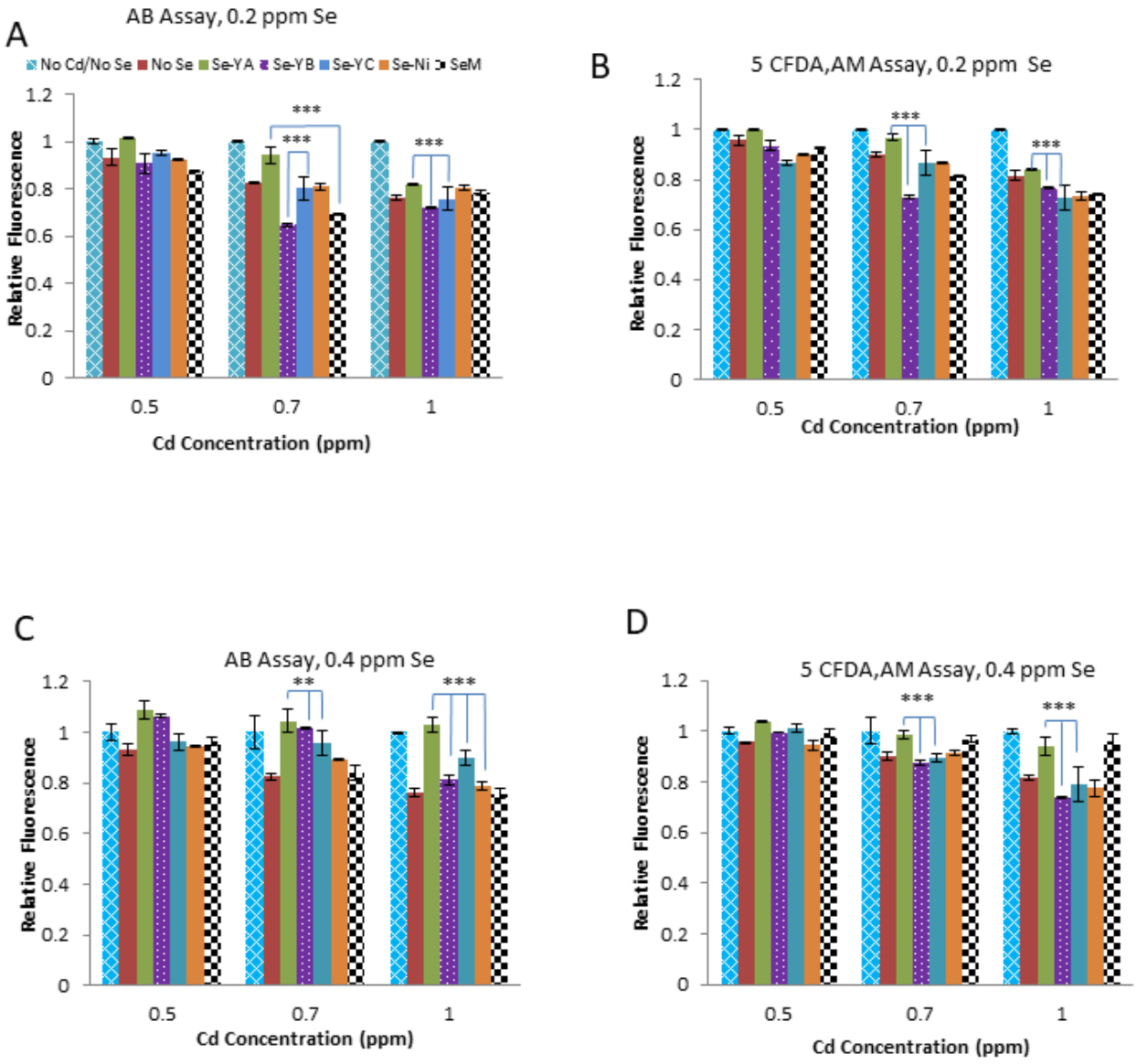

Figure 3.1: Effect on IPEC-J2 cells of pre-incubation with Se sources (0.2 ppm, A and B; 0.4 ppm C and D) compounds for 48 h prior to challenge with $CdCl_2$. Shown are Alamar Blue (graphs **A** and **C**) and CFDA-AM assays (graphs **B** and **D**). The $CdCl_2$ concentrations used (ppm) are given as numbers below each graph. The bar colour codes in **A** apply to all four charts. Data are expressed as the mean ± SD of triplicate samples and the results for each assay are presented as relative to the corresponding Se-treated unchallenged controls (not shown) which were set as 1. Significant differences were determined by one-way ANOVA (denoted as **P<0.01 and ***P<0.001) and highlight comparisons between the data points indicated. Asterisk represents significant difference between Se sources.

3.3.2) Se-Y sources differ in their capacity to protect IPEC-J2 cells from Cd-induced DNA damage

The potential of different Se-Y sources to protect IPEC-J2 cells from Cd-induced single- and double-stranded DNA damage was investigated using the alkaline comet assay. When used at 0.2 ppm Se, none of the Se-Y sources impacted on the extent of Cd-induced DNA damage in the absence of Se, as determined using the percent DNA criterion, though an increase in damage was observed in the presence of Se-Ni (Fig. 3.2 A). At 0.4 ppm Se, a clear difference in the relative protective performances of the Se-Y sources emerged, in the order Se-YA>Se-YB>Se-YC (Fig. 3.2 B) with Se-YA affording a significant protective effect when cells were exposed to 0.7 ppm Cd. Supplementation with Se-YB, Se-YC or Se-Ni at 0.4 ppm did not impact on the DNA damaging effects seen under these conditions.

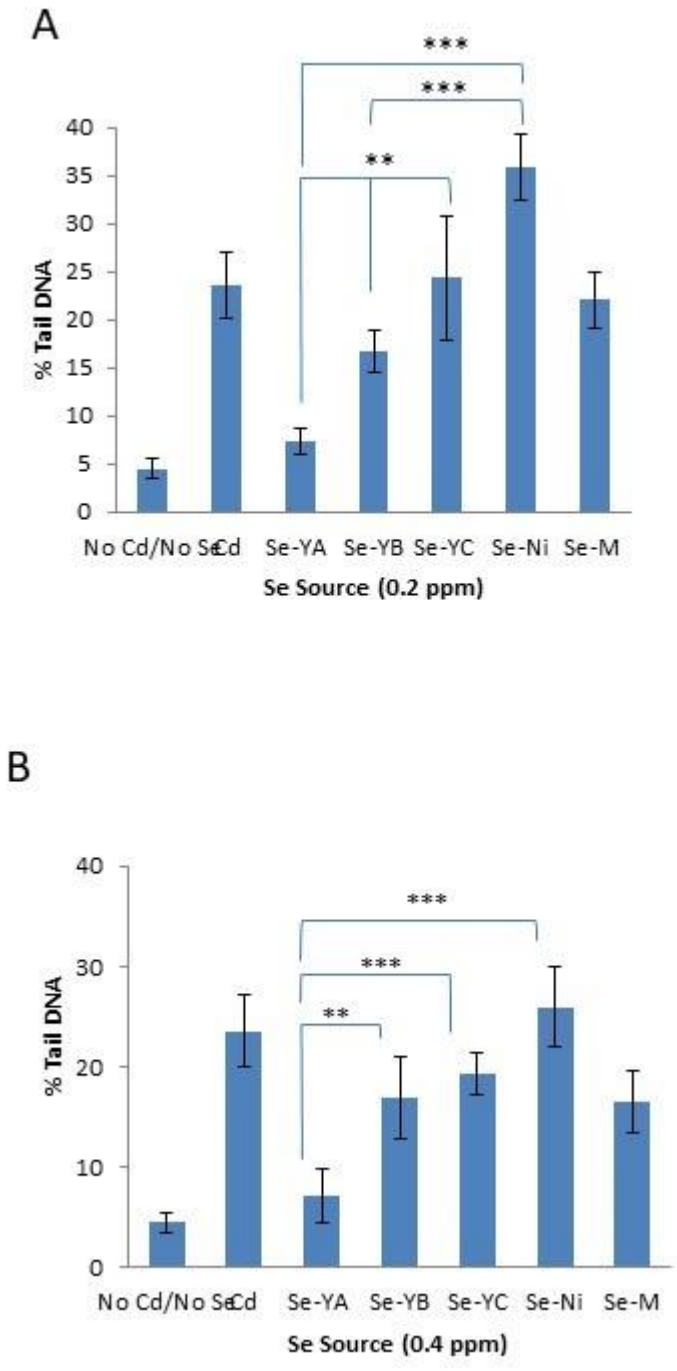

Figure 3.2: Determination of DNA damage in IPEC-J2 cells by Alkaline comet assay following challenge with $CdCl_2$. Cells were first pre-incubated for 48 h with various Se sources (X axis labels) then treated with Cd (0.7 ppm) for a further 24 h. Comet data from the parameters indicated on the Y axis of each graph were determined using OpenComet software. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (denoted as *P<0.05, **P<0.01 and ***P<0.001) and highlight comparisons between the data points indicated. Asterisk represents significant difference between Se sources.

In the TUNEL assay, the extent of TUNEL staining reflects the level of nicked DNA usually associated with DNA fragmentation and the initiation of programmed cell death (apoptosis). Here, it can be seen that supplementation with Se-Y sources at 0.2 ppm prior to challenge with Cd correlated with significantly less apoptosis when compared to the corresponding no-Se control cells (Fig. 3.3, labelled as "Cd"). At 0.4 ppm Se, this protective effect was only visible in the case of Se-YA. In contrast, no significant changes to non-apoptotic IPEC-J2 populations were evident when Se-YB or Se-YC were used in the same experiment. Se-Ni and Se-M pretreatment resulted in significantly higher apoptotic populations at both Se concentrations tested. Overall, the comet and TUNEL data showed that the extent of protection from Cd-induced DNA damage and apoptotic fragmentation varied considerably with different Se-Y sources, with Se-YA affording greater protection than Se-YB and Se-YC when EFSA-recommended supplementation/total Se levels were used, and Se-Ni and Se-M enhancing the Cd-induced DNA apoptosis.

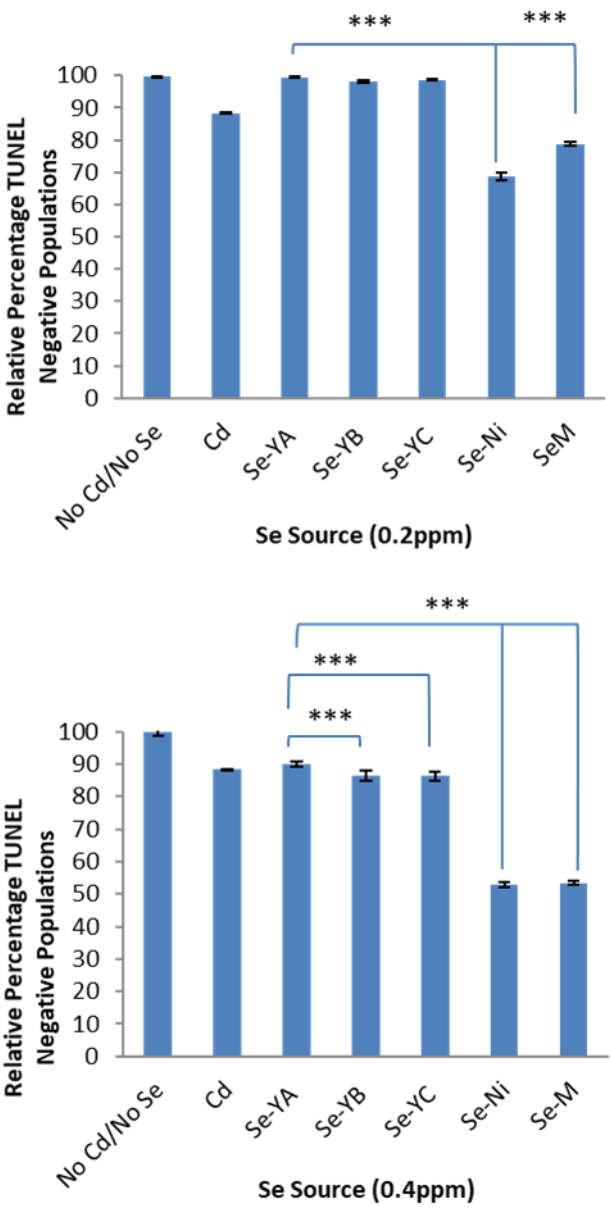

Figure 3.3: TUNEL analysis of IPEC-J2 cells following 48 h pre-incubation with Se sources and subsequent exposure to $CdCl_2$ (0.7 ppm) for 24 h. DNA damage was induced in IPEC-J2 using $CdCl_2$ at 0.7 ppm and TUNEL-Negative populations are presented as a fraction of the same group in the corresponding $CdCl_2$- treated/no Se sample. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (denoted as ***P<0.001) and highlight comparisons between the data points indicated. Asterisk represents significant difference between Se sources.

3.3.3) Organo-selenium sources moderate Cd-induced oxidised damage to DNA bases

The IPEC-J2 model was also used to assess oxidised damage to DNA bases following $CdCl_2$ insult and to compare the potential contributions of Se supplementation sources in this regard. The concentration of Se used for this analysis was 0.4 ppm as it was under these conditions that differences between Se sources, in terms of their capacity to prevent DNA damage, were most pronounced. In the modified comet assay, the types of oxidised bases present may be inferred from the substrate specificity of the DNA repair enzymes used, in this case Endo III and FPG. These lesion-specific DNA glycosylases remove oxidised damaged purines and pyrimidines, respectively, from double-stranded DNA leaving a one base gap which is then converted to a break by the high pH used during subsequent steps. IPEC-J2 were grown for 48 h with/without Se supplementation (0.4 ppm) and treated with 0.7 ppm Cd for 24 h. Agarose-embedded nucleoids were then prepared as per comet assay and incubated with either FPG or Endo III prior to the electrophoresis step. Increased levels of oxidized damaged bases (net enzyme-sensitive sites) were evident in all cases following Cd treatment, as indicated by increased comet percent tail DNA values after treatment with each enzyme (Fig. 3.4 A, B). In the case of supplementation with Se-YA, both the basal levels of oxidised damaged sites and the extent of net enzyme-sensitive sites were seen to decrease when either enzyme was used. This was not the case when other Se sources were used; indeed when Se-Ni was used as supplement the preponderance of both basal and net enzyme sensitive sites was seen to rise significantly following Cd insult. This data highlighted variations in the capacity of different Se-Y sources to mitigate against Cd-induced oxidized damage to DNA bases.

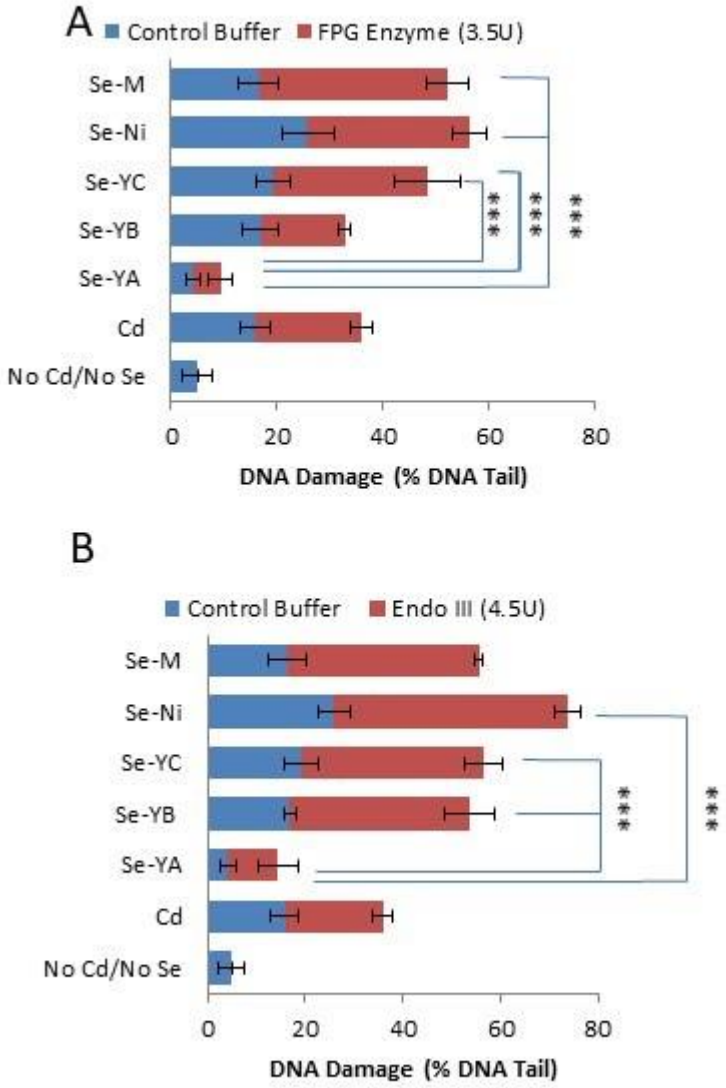

Fig. 3.4 Se sources moderate oxidized damage to DNA bases. The enzymes FPG (a) and Endo III (b) were used to assess DNA base damage in IPEC-J2 cells that had been pre-incubated for 48 h with Se sources (0.4 ppm), as indicated on the Y axes, and subsequently exposed to 0.7 ppm CdCl2 for 24 h. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (denoted as ***P\0.001) and highlight comparisons between the data points indicated. Asterisk represents significant difference between Se sources.

3.3.4) DNA repair activity in Se-supplemented IPEC-J2 following insult with Cd

The effect of Se supplementation on DNA base excision repair (BER) activity in IPEC-J2, following Cd insult, was next investigated. BER of oxidised base damage is effected through a series of enzymatic reactions, whereby as a first step endogenous cellular glycosylases excise the oxidised damaged base prior to nucleoside removal and substitution with an intact nucleotide. Here, substrate nucleoids were prepared from Cd treated IPEC-J2 cells and incubated with cell-free extracts (containing endogenous DNA-repair enzymes) made from IPEC-J2 that had been grown for 48 h in Se-supplemented medium. Following electrophoresis, the percent comet tail DNA observed is proportional to the DNA repair incision activity of the corresponding extract. Preliminary experiments were carried out using different concentrations of each cell-free extract in order to establish the optimum concentrations to use (data not shown). This challenge study allowed comparison of the relative effects of each Se source used on DNA repair activity. It can be seen that supplementation of IPEC-J2 with Se-YA correlated with significantly enhanced DNA repair activity relative to all other Se sources, DNA repair activity demonstrated by Se-YA supplementation was significantly increased in comparison to Se-YB and Se-YC. Repair activity of Se-M was seen to be comparable to Se-YB. To accurately determine repair-related DNA incisions a number of experimental controls were implemented: 1) non-exposed substrate cells incubated with buffer were included as a background control to signify damage caused during cell preparation and handling. 2) Cd-induced DNA damage was determined using exposed cells incubated with buffer, and 3) non-specific endonuclease activity of the extract was indicated by non-exposed cells treated with extract. Repair-related DNA incisions were calculated using the formula:

Net DNA repair activity = (Cd-induced DNA incisions $_{\text{Se exposed cell extract/buffer incubation}}$)

- (undamaged cell DNA incisions $_{\text{Se exposed cell extract/buffer incubation}}$)

- (Cd-induced DNA incisions $_{\text{no Se exposure cell extract/buffer incubation}}$).

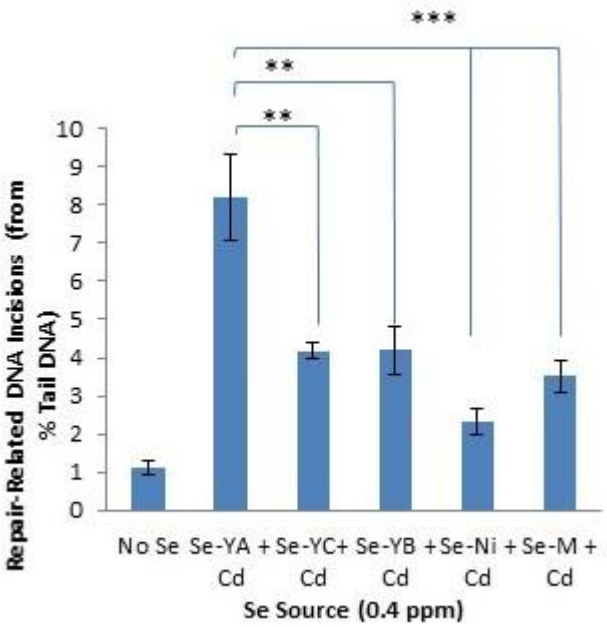

Fig. 3.5: DNA repair activity in Se-Y treated IPEC-J2. The figure shows data obtained following an in vitro assay for Base Excision Repair (BER). IPEC-J2 cell extracts were prepared 48 h following supplementation with Se sources and assessed for BER using Cd-insulted (0.7 ppm) IPEC-J2 as substrate cells. 'No Se' refers to cells with Cd but without Se. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (**P\0.01 and ***P\0.001) and highlight comparisons between the data points indicated. Asterisks represents significant difference between Se sources.

3.3.5) Anti-oxidant enzyme activity following Se supplementation and challenge with Cd

Thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) are key antioxidant seleno-enzymes that modulate of the intracellular redox environment and protect cells from oxidative stress, including damage to DNA bases, initiated by excess ROS. Here, TrxR and GPx activity levels in IPEC-J2 were assessed following supplementation with Se sources and in each case also following challenge with Cd. Elevated basal levels of TrxR activity were observed following supplementation with all three Se-Y sources at 0.4 ppm Se (Se-YA, Se-YB and Se-YC) and 2/3 (Se-YA and Se-YB) at 0.2 ppm Se (Fig. 3.6 E, G). In contrast, TrxR levels were significantly lower when either Se-Ni or Se-M were used at both Se concentrations. In the absence of Se supplementation, increased TrxR levels were also observed in response to treatment with Cd (Fig. 3.6 F,H). Importantly however, only in the case of supplementation with Se-YA were TrxR levels maintained, and in fact increased, relative to the corresponding +Cd/-Se control. In all instances, TrxR activity levels decreased in response to Cd treatment, an effect that was most pronounced following supplementation with inorganic Se at 0.4 ppm. In the case of GPx, basal activity levels rose following Cd treatment and also in the absence of Cd during supplementation with all Se sources used at both 0.2 and 0.4 ppm (Fig. 3.6 A-D). All Se sources maintained GPx activity at or above +Cd/-Se basal levels following Cd challenge. Of the three Se-Y sources used, Se-YA was the most effective at maintaining GPx activity levels (at both 0.2 and 0.4 ppm Se) following Cd insult and results here once more followed the trend of Se-YA>Se-YB>Se-YC.

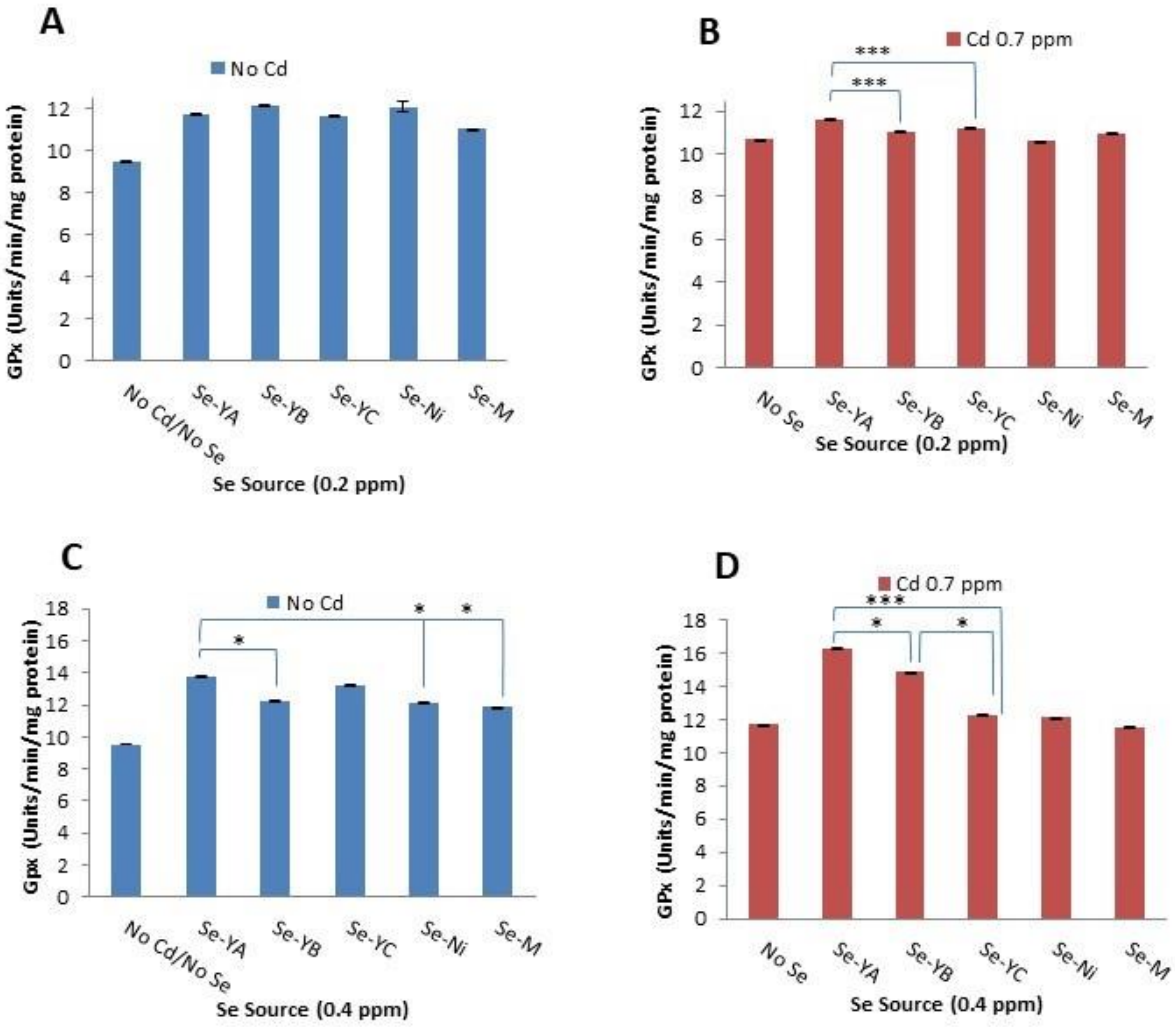

Figure 3.6 (i): Glutathione peroxidase (GPx) enzyme activity following Se supplementation (a, c) and Se supplementation and challenge with Cd (b, d). GPx (a–d) activities were determined in extracts prepared from IPEC-J2 cells that had been pre- incubated for 48 h with Se sources (X axis labels) and assayed or subsequently exposed to 0.7 ppm $CdCl_2$ for 24 h prior to analysis. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (*P\0.05 and ***P\0.001) and highlight comparisons made with the corresponding No Cd/No-Se (blue) or No Se sample (red). Asterisk represents significant difference between Se sources.

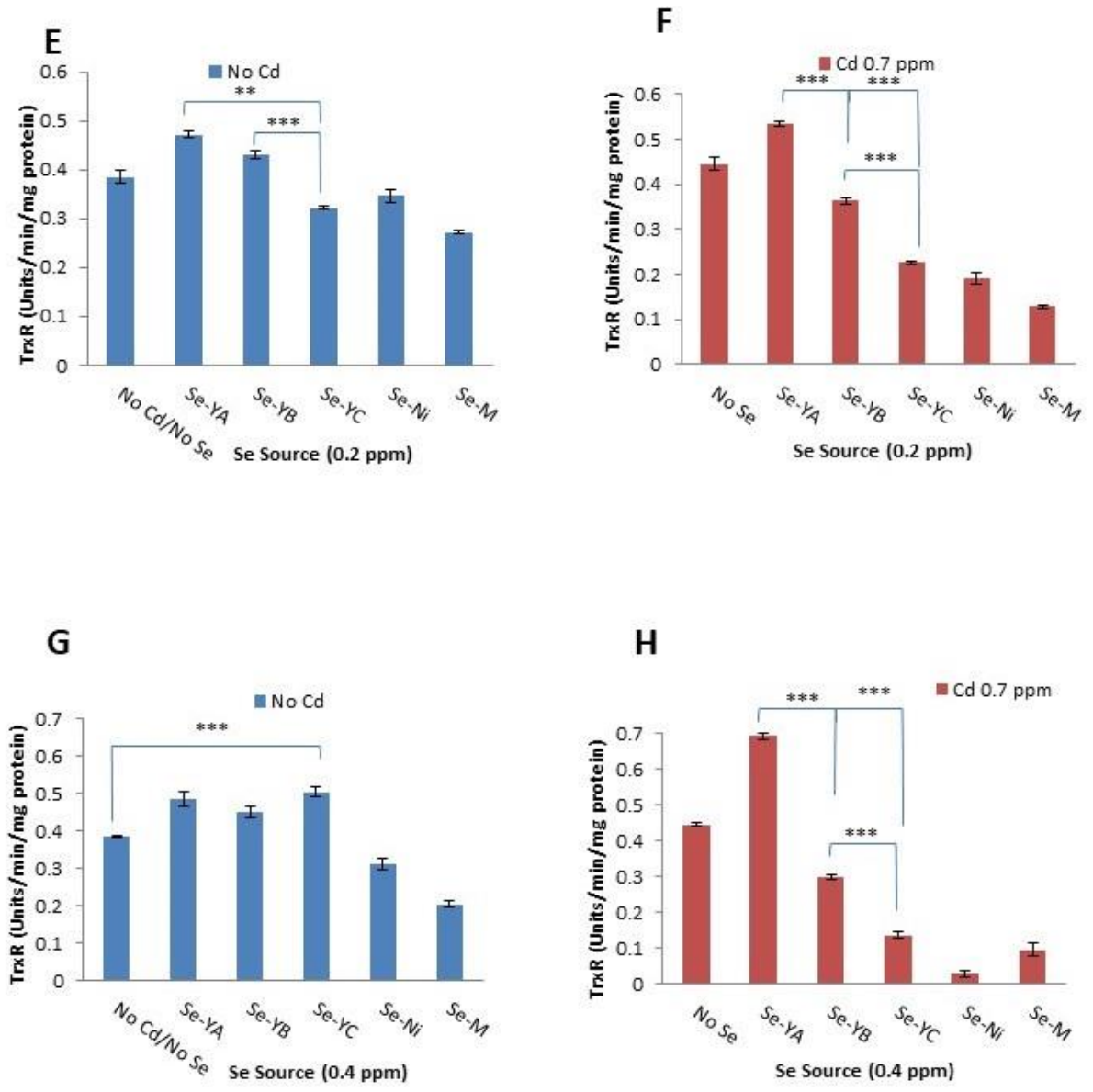

Figure 3.6 (ii): Thioredoxin reductase (TrxR) enzyme activity following Se supplementation (e, g) and Se supplementation and challenge with Cd (f, h). TrxR (E–H) activities were determined in extracts prepared from IPEC-J2 cells that had been pre- incubated for 48 h with Se sources (X axis labels) and assayed or subsequently exposed to 0.7 ppm CdCl2 for 24 h prior to analysis. Data are expressed as the mean ± SD of triplicate samples. Significant differences were determined by one-way ANOVA (**P\0.01 and ***P\0.001) and highlight comparisons made with the corresponding No Cd/No-Se (blue) or No Se sample (red). Asterisk represents significant difference between Se sources.

3.4) Discussion

The biological efficacy of Se-Y is crucially dependent on the digestibility and accessibility of Se-containing proteins and peptides and not just on the level of Se-M (13, 37). When focusing on the physiological effects of Se-Y preparations *in vitro*, a digestion model that simulates gastric and intestinal digestion would be likely to best reflect the bioavailability of organic Se *in vivo*. Although there are differences amongst published models, enzymes are of major importance and both pepsin and pancreatin are used sequentially to replicate the gastric and small intestine environment, respectively (27), hence the protocol used in the present study.

Significant differences between Se-Y sources emerged under supplementation conditions of 0.4 ppm total Se and 0.7 or 1 ppm Cd challenge. Analysis of the effects of Se on Cd induced cell injury, DNA damage and apoptosis, revealed either no protective effect or even worsening effects of the Se-Y sources assayed. An appreciable disparity in the relative performances of the Se-Y sources was observed, in the order Se-YA>Se-YB>Se-YC. At 0.4 ppm Se in all assays conducted Se-YB and Se-YC afforded no protection against Cd challenge. At both Se concentrations and in all assays implemented, Se-YC either failed to protect against Cd challenge or enhanced the negative effects of Cd-induced damage. Se-M afforded a greater ameliorative effect compared to Se-YB/C against Cd-induced DNA damage and apoptosis, However, the effect of Se-M was not as significant as the protection afforded by Se-YA.

FPG and Endo III are known to play prominent roles in global DNA base excision repair *in vivo*, and although their substrate specificities partially overlap *in vitro*, their activities here nonetheless reflect the extensive damage to purines and pyrimidines due to Cd insult. The data shown revealed significant variations in the capacities of different Se-Y sources to mitigate against Cd-induced 133xidized damage to DNA bases as outlined above in Fig. 3.4. These

differences potentially reflected either a reduction in the extent of damage occurring due to Cd, or the enhanced repair of oxidised DNA bases, or both mechanisms.

The potential of different Se sources to modulate cellular DNA repair activity in IPEC-J2 was investigated. Regarding Se-Y sources, the data showed a significant enhancement of BER capability only in the case of Se-YA, again highlighting important variations in the performance of different supplementation sources. The *in vitro* repair assay used to assess the potential BER capacity of Se cell extracts on Cd exposed IPEC-J2 cells in this study is a novel biochemical approach to DNA repair analysis (38). To date, the *in vitro* repair assay has seldom been used in DNA repair studies and has not been implemented in previous literature exploring the DNA repair capacity of Se-Y sources thus adding to the innovative aspect of this paper. Previous studies have indicated that Se plays a significant role in DNA repair (18, 21, 39, 40). One such study demonstrated activation of BER in response to the organic selenium (Se-M) and involved p53-dependent interaction of Gadd45a with repair proteins(41) while proliferating cell nuclear antigen (PCNA) binding assays have also suggested an upregulation of BER following SeM treatment (42).

Se is an essential component of GPx, TrxR and other antioxidant enzymes. Increased activity levels of these enzymes is known to correlate with an enhanced cellular capacity to scavenge ROS species and to prevent 134xidized damage to biomacromolecules (43). As the total Se concentrations used were the same, irrespective of the Se source, it can be concluded that the variations in anti-ROS enzyme activities seen in response to the different Se-Y formulations reflect the differential bioavailability of organic Se in each case as shown in Fig. 3.6.

This study has provided evidence of important differences between Se-Y sources in terms of their capacity to protect cells from Cd-induced damage. The differences observed between Se-Y preparations may be the result of one or a combination of factors: (i) the use of different strains of *S. cerevisiae*, (ii) the profiles, digestibility and accessibility (i.e. bioavailability) of

seleno-amino acids and peptides in each Se-Y preparation, and (iii) the potential loss of some Se species due to leaching that can occur during post-production and prior to drying. Se-Y production methods in which propagation conditions are carefully controlled can generate yeast with levels of Se exceeding 2000 ppm with little or no adverse effects on protein synthesis or yeast growth characteristics (44). The biosynthesis of yeast organo-selenium compounds is thought to be achieved through well-characterised biochemical pathways leading to organo-sulphur biosynthesis, to include principally compounds such as Se-M, seleno-cysteine and seleno-cystine, with smaller amounts of seleno-homocystenine, seleno-cystathione, methyl-selenocysteine, S-adenosyl-seleno-methionine, seleno-trisulphide compounds, seleno-gluthatione and various seleno-thiols (13, 44). Such seleno-amino acids can then be incorporated into protein molecules. It is likely that inherent differences between yeast strains are a major contributory factor to the differential synthesis and deposition of such Se compounds within yeast fractions. Variations in Se-Y production methods may also play a role in this regard.

In conclusion, Se-Y has economic and environmental advantages over traditional inorganic Se. Increased bioavailability means lower quantities of Se are required to supplement Se-deficient animal feeds. This translates directly into cost savings and a reduction in the level of unabsorbed Se excreted by the faecal route, a significant advantage where intensive farming techniques are employed. The results presented in this study have highlighted important differences in the bio-efficiency of different commercially available Se-Y preparations in terms of their ability to enhance a range of cellular mechanisms that protect porcine gut epithelial cells from damage due to Cd exposure.

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Chapter Four: Selenium sources differentially modulate gene expression in porcine jejunal epithelial cells following Cd exposure.

Abstract:

Cadmium (Cd) is a relatively abundant bio-toxic metal that indirectly produces reactive oxygen species (ROS) by the displacement of Fe^{2+} and Cu^{+} ions (1). Selenium (Se) possesses antioxidant properties and has exhibited a protective effect against DNA damage. However, further work is needed to elucidate the role of Se in Cd-induced cytotoxicity and the varying effects of different Se compounds. In this present study RNA-sequencing (RNA-seq) technology and transcriptome analysis were implemented to detect potential differential gene expression between a range of Se sources to determine their effect on Cd-induced toxicity. Having previously observed phenotypic changes in DNA damage between cells treated with different Se sources and exposed to Cd (2, 3), gene expression analysis was performed in an attempt to elucidate the genetic mechanisms of Se in response to Cd exposure. The first aim of this study was to identify differentially expressed genes in IPEC-J2 cells between Cd exposed cells and non-exposed cells. The second objective was to assess the differences in gene expressions between the Se treated samples following exposure to Cd. In this study, three Se-yeast (Se-Y) compounds were analysed, Se-YA, Se-YB and Se-YC, and one inorganic and one organic Se compound, sodium selenite (Se-Ni) and selenomethionine (Se-M), respectively. Porcine jejunal epithelial cells (IPEC-J2) were pre-incubated for 48 h with one of the four Se compounds and subsequently exposed to Cd. The differential gene expression results presented in this study confirm that Cd exposure alone significantly increases stress response pathways. There were no differences in gene expression detected between Cd/No Se and pre-treatments with either Cd + Se-Ni or Cd + Se-M. The gene expression analysis also highlighted differences in gene expression profiles between the organic Se-Y compounds. Se-YA and Se-YC provided the highest level of protection against Cd-induced cellular stress among the organic Se-Y

compounds. The differentiation in gene expression pattern among the three Se-Y compounds provides further support for previous studies that indicated a difference in bio efficiency between various Se-Y preparations.

4.1) Introduction:

The Human Genome Project transformed biology by providing a complete genetic blueprint for human life as well as delivering whole genome sequences for several organisms that play a major role in scientific research such as the mouse and the fruit fly (4). It utilised high throughput DNA sequencing instruments based on capillary electrophoresis (5). Automated capillary electrophoresis-based sequencers (Sanger method) became the main technology used in the National Institutes of Health (NIH) and Celera led Human Genome Projects, and this method of DNA sequencing has become known as "first generation sequencing" (6). The sequencing of the human genome encouraged the advancement of sequencing technologies. The introduction of a variety of massively paralleled sequencing instruments began in 2005. These sequencers were vastly different from the capillary electrophoresis sequencing methods used previously and marked the commencement of "Next Generation Sequencing" (NGS) in genomic science (7). While each NGS instrument has its own individual characteristics, all sequencing technologies share a number of common features including 1) template preparation, 2) amplification of library fragments, 3) automatic sequencing reactions and detection, 4) data analysis. The distinctive protocols denoted for each unique sequencing instrument determines the type of data generated from each sequencing technology and differentiates one sequencing platform from another (4, 7). The past decade has seen the emergence of several different NGS platforms each with their own advantages and disadvantages. The work in this chapter was carried out using the NGS platform Illumina sequencing by synthesis (SBS) technology as this

is the predominantly used sequencing technology in both clinical and research applications and currently generates ninety per cent of sequencing data produced worldwide (8). Although NGS technology is comprised of several different sequencing platforms as, here the term NGS will refer to solely to Illumina SBS technology. Although NGS technology is largely superseding Sanger CE based sequencing in molecular diagnostics, the basic concept behind the two platforms is comparable. Fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) are incorporated into the template DNA strand by a polymerase during consecutive cycles of DNA synthesis. At the point of incorporation of the fluorescently labelled dNTPs, the nucleotides are identified by fluorophore excitation (9). An NGS experiment can yield more than 250 million unique reads, while a Sanger reaction returns a single DNA sequence (10). The NGS workflow consists of four stages (see Fig. 4.1):

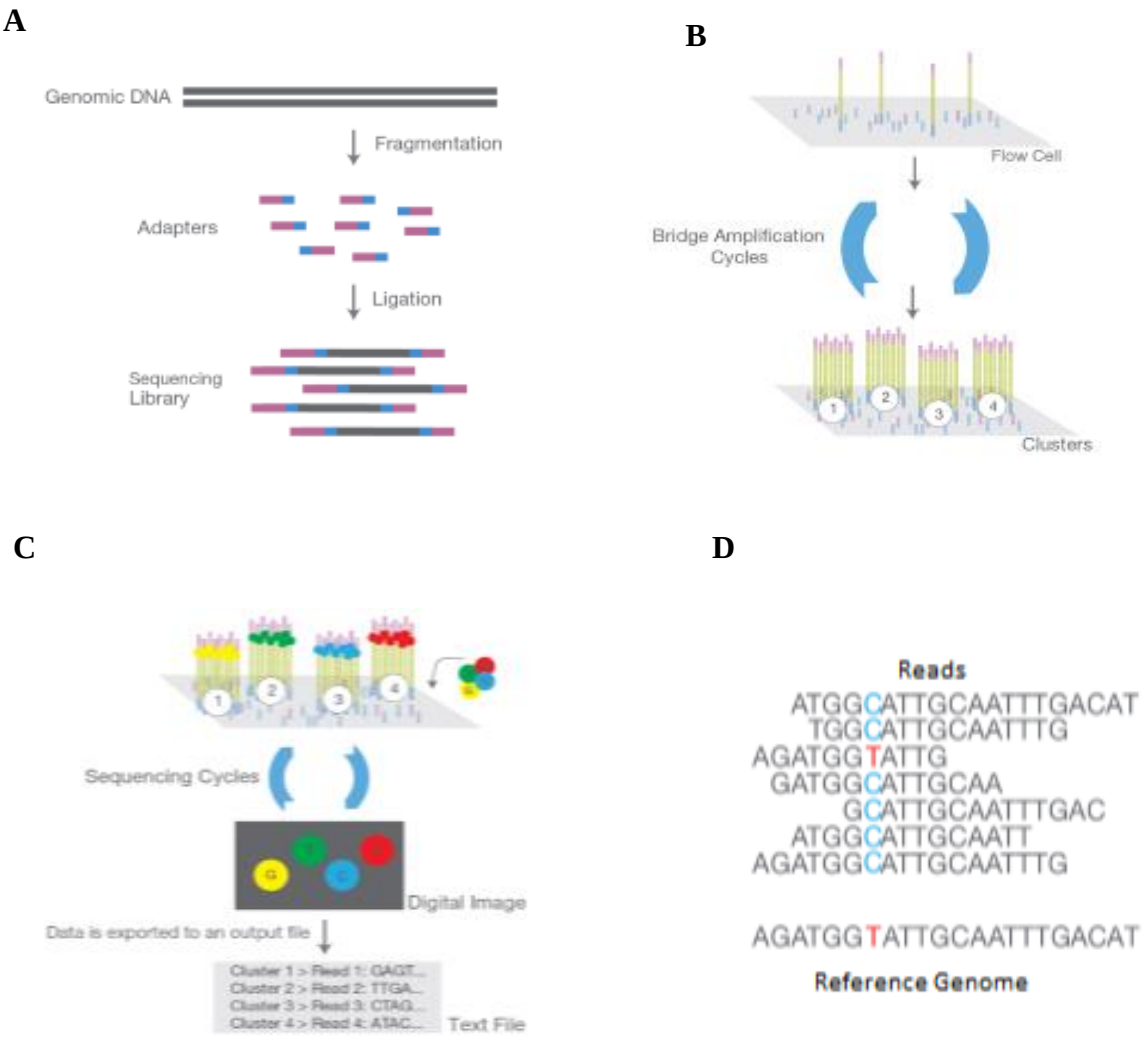


Figure 4.1: The four phases of the NGS workflow (A-D). Library preparation (A), cluster amplification (B), sequencing (C), alignment and data analysis, adapted from (8).

Fundamental to the NGS workflow is the conversion of nucleic acid material into a sequencing library. A comprehensive suite of library preparation protocols exists; however, the different procedures largely follow the same set of steps: 1) fragmentation of DNA or RNA molecules, 2) generation of double stranded DNA, 3) oligonucleotide adapter ligation and PCR amplification, 4) quantitation of the final library product (11, 12). The adapter-modified, single stranded DNA is added to the flow cell where the fragments are captured on a lawn of surface-bound oligos that are complementary to the library adapters. The DNA templates in this SBS platform are amplified by “bridge” amplification, wherein the immobilised DNA strands arch

over and attach to the adjacent oligonucleotide by hybridization. Following multiple sequential cycles, each fragment has been clonally amplified resulting in cluster formations with each clonal cluster containing approximately 1000 molecules (13). The clusters are optically analysed and the fluorescent intensity is recorded (8, 13, 14). Following sequencing, the reads can be aligned to the reference genome allowing identification of the newly sequenced reads. The power of an RNA-seq differential expression experiment to detect significant differences relies on its ability to distinguish true biological differences between conditions from the variability that occurs in repeated measurements of the same condition (15). For this reason, experimental design in RNA-seq studies is of vital importance in order to obtain accurate data that is representative of true differential gene expression. There are several experimental design factors that need to be considered before the commencement of an RNA-seq study. These include selection of an appropriate sequencing platform (16), library preparation (17), biological replication (18), sequencing depth (19).

Transcriptomic analysis has been implemented to study the effects of Se supplementation on Cd-induced damage. One such study analysed the mRNA levels of selenoprotein genes and inflammatory factors by RT-qPCR in chicken kidney tissue following Se supplementation and Cd exposure. It was observed that Se ameliorated the Cd-induced increase in the levels of inflammatory factors (prostaglandin E synthase, nuclear factor-kappa B, tumor necrosis factor-α) and alleviated the Cd-induced downregulation in the levels of 25 selenoprotein genes (20). The effect of Se and Cd exposure on the transcription of heat shock protein (HSP) 27, HSP40, HSP60, HSP70 and HSP90 from chicken splenic lymphocyte mRNA was analysed. Treatment of the lymphocytes with Se alongside Cd increased the mRNA expression of HSPs which were reduced by Cd treatment. The results suggest that one of the ways in which Se may have provided protection against Cd-induced toxicity was through the stimulation of the levels of HSPs (21). Transcriptomic analysis of the effect of Cd exposure in freshwater crab revealed an

upregulation following Cd exposure in the oxidative phosphorylation pathways, detoxification pathways and the anti-oxidant defense system. A downregulation of immunity related genes was observed following Cd insult and the alteration in gene expression profiles was concentration dependent (22). The potential protective effect of Se on Cd-induced autophagy was assessed using RT-qPCR to analyse mRNA gene expression levels. The autophagy related genes (*Beclin1, LC3-I, LC3-II, Atg5*) were upregulated in response to Cd exposure in chicken pancreatic cells, whereas a downregulation of these genes were observed in cells that were subjected to both Se and Cd treatments (23). Transcriptomic data from a recent study demonstrated that Se supplementation and subsequent Cd exposure resulted in a significant reduction in the toxic effects of Cd in the mouse model. Se supplementation inhibited the Cd-induced dysregulation of inflammatory, metabolic and amino acid metabolism pathways (24). Another study utilised transcriptomic and proteomic technologies to analyse the potential role of dietary Se in colorectal cancer prevention. The results of the RNA sequencing analysis performed on patient biopsy samples showed a downregulation of inflammatory and immune response pathways in the suboptimal Se status group, which may increase cancer risk. Overall there was differential expression of 254 cancer associated genes between the optimal and suboptimal Se status groups. Furthermore, 40% of inflammatory associated genes and 50% of cell death related gens were differentially expressed between the two treatment groups (25).

In this present study the potential effects of a range of Se sources on Cd-induced toxicity were evaluated by utilising RNA-seq technology and transcriptome analysis to detect differential gene expression. Gene expression analysis was carried out to attempt to identify genetic mechanisms at play having seen phenotypic changes in DNA damage between cells treated with different selenium sources and exposed to cadmium (2, 3). The main objectives therefore were firstly to identify differentially expressed genes in IPEC-J2 cells between Cd exposed

cells and non-exposed cells and secondly, to evaluate the differences of gene expressions between the Se treated samples following exposure to Cd.

4.2) Materials and Methods:

4.2.1) Cell Culture and treatment conditions:

IPEC-J2 Porcine jejunal epithelial cells (IPEC-J2, DSMZ Braunschweig, Germany) were grown in Dulbecco's Modified Eagle's/Ham's Nutrient Mixture F-12 medium (DMEM/Nutrient Mixture F-12 Ham) supplemented with 10% porcine serum and 1% penicillin-streptomycin at 370C in a humidified 5% CO_2 atmosphere (Galaxy S CO2 Incubator, Model No:170-200, RS Biotech Laboratory Equipment Ltd., Irvine, United Kingdom). Cells were passaged just prior to confluence every 3 to 4 days following removal with trypsin/EDTA (all cell culture products were from Sigma Aldrich). All manipulations were carried out in a Class II biological safety cabinet (BioAire Aura 2000 BS; Bioair Instruments, Pavia, Italy). IPEC-J2 cells are an established non-transformed *in vitro* gastrointestinal model from which results can be extrapolated to the *in vivo* situation. Here, IPEC-J2 cells were cultivated in the presence of porcine serum (PS) as opposed to conventional foetal bovine serum FBS. This species-specific growth medium supplementation has been shown to promote the growth of IPEC-J2 which are much more similar in terms of cell architecture, morphology, transport functions and trans-epithelial resistance to primary pig jejunocytes (26). Cells were incubated in the presence or absence of the individual Se sources at a Se concentration of 0.4 ppm and incubated at 37^{o}C for 48 h. They were then insulted with 0.7 ppm cadmium chloride ($CdCl_2$) and incubated for a further 24 h at 37^{o} C. Stock solutions (5 mM) of $CdCl_2$ were prepared in MilliQ-H2O and then diluted in serum free growth medium to achieve these desired final

concentrations. Three replicates were produced for each sample, each replicate was prepared on separate days (n=3).

4.2.2) Selenium compound preparation

Selenium extracts were prepared from three different commercially available sources of selenised yeast (Se-YA, Se-YB and Se-YC), sodium selenite (Se-Ni) and selenomethionine (Se-M). The yeast products were sourced from different manufacturers who generate dried and inactivated preparations from different strains of *Saccharomyces cerevisiae* (27). Extracts were prepared using a porcine digestion model (28) and experimental volumes outlined elsewhere (29) as follows: 500 mg of each yeast powder was placed in separate 50 mL polystyrene tubes. 5 mL of sodium phosphate buffer (0.1 M, pH 6) was added to each along with 2 mL of 0.2 M HCl and the pH was adjusted to 2.0. Pepsin (Sigma Aldrich) and chloramphenicol (Sigma Aldrich) were then added to final concentrations of 6 Units/mL and 0.3 mg/mL, respectively. Digestions were incubated for 2 h with gentle shaking at 39^0C. Two mL of sodium phosphate buffer (0.2 M, pH 6.8) and 1 mL of 0.6 M NaOH were then added to each tube and the pH was adjusted to 6.8. Pancreatin solution (Sigma Aldrich) was added to a final concentration of 3mg/mL and the tubes were then incubated for 4 h with gentle shaking at 39^0C. Tubes were then centrifuged at 16,000 x g for 15 min at 4^0C. The supernatants obtained were transferred to ultrafiltration tubes with a molecular weight cut-off of 10,000 Daltons (Sartorius, Gottingen, Germany) and these were centrifuged at 8,000 x g for 3 h at 4^0C. Following centrifugation, the filtrates obtained were aliquoted into microfuge tubes and stored at -70^0C until use. Total Se concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent Technologies, Maynooth, Ireland). All Se-Y preparations were aseptically filtered (0.25 μm) and diluted in DMEM/Nutrient Mixture F-12 Ham to final Se concentrations

of either 0.2 ppm or 0.4 ppm immediately prior to use in cell culture. Solutions of Se-M and Se-Ni were prepared in MilliQ-H_2O and processed using the porcine digestion model as described above.

4.2.3) RNA extraction and Bioanalyzer analysis:

Following pre-incubation with Se sources and subsequent exposure to $CdCl_2$, cell culture media was aspirated from each of the flasks and carefully washed with 5mL PBS. Cells were then incubated with 5mL of trypsin for 10 minutes, following cell detachment the cell suspension was centrifuged at 1000rpm for 15 minutes. The supernatant was aspirated, and the cell pellet was resuspended in 5mL of media, a 10µL aliquot of the cell solution was added to 100µL of Trypan blue (Sigma Aldrich, Co. Wicklow, Ireland) and the cell count was calculated using a hemocyotometer (Brightline). The cell solution was aliquoted into separate tubes each containing approximately 4 x 10^6 cells per tube, the cells were centrifuged, the media was removed, and the cell pellets were used immediately in the RNA extraction procedure. RNA was extracted from IPEC-J2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions including an on-column DNase digestion with no modifications and the subsequent RNA was stored at -80^{o}C until further use. Preliminary purity and concentration readings for each RNA sample was determined using the Nanodrop 2000 (Fisher Scientific, Ballycoolin, Dublin). Final RNA quality and concentration was then assessed using the RNA 6000 Nano Kit (Agilent, Maynooth, Ireland) and Agilent 2100 bioanalyzer conducted as per manufacturer's instructions with no deviations. All samples had an RNA integrity number (RIN) of 10.

4.2.4) Library Preparation and Sequencing:

A total RNA concentration of 1µg of each sample was used as the starting material in the library preparation procedure. The RNA library was prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Hitchin, UK) and was used in conjunction with the NEBNext Poly(a) mRNA Magnetic Isolation Module (NEB, Hitchin, UK) according to manufacturer's instructions to isolate mRNA from total RNA (Fig. 4.2). The quality of the resulting cDNA libraries was assessed using the High Sensitivity DNA Kit and Agilent 2100 bioanalyzer.

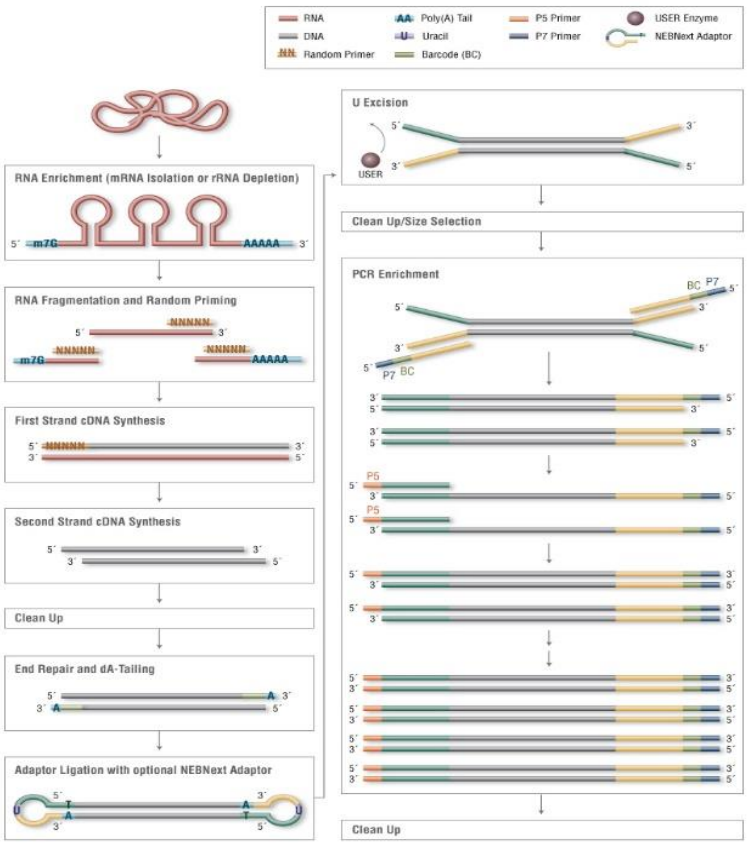


Figure 4.2: NEBNext Ultra II Directional RNA Library Prep Kit procedure (30).

The MiSeq Reagent Kit v2 was used to denature the libraries prior to sequencing as per manufacturer's instructions. Briefly, each library was diluted to a final concentration of 2nM prior to denaturation with 0.2N NaOH and HT1 (hybridization buffer) was added resulting in a 10pM denatured library. The 10pM library was subsequently spiked with a 1% PhiX control and the libraries were loaded onto the Miseq reagent cartridge and the 76-cycle single read sequencing run was set up.

4.2.5) Gene Expression Profile Analysis:

All data analysis was carried out using BaseSpace Labs software (BaseSpace Sequence Hub-version 5.6.0, Illumina) (31). Following sequencing, read quality was assessed using FASTQC software (version 1.0.0, Illumina). Raw reads were trimmed by removing adaptor sequences, subtracting bases on both the 5' and 3' ends of the reads and filtering read length, this was performed using the FASTQ Toolkit (version 2.2.0, Illumina). The subsequent sequences were then exported to the RNA-Seq alignment application (version 1.1.1, Illumina) for genome mapping to the *Sus scrofa* reference genome (Pig) as provided by UCSC (susScr3, Aug. 2011). Differential gene expression analysis was conducted using Cufflinks assembly and differential expression (version 2.1.0, Illumina). See Table 4.2 below for parameter details and description of the relevant application functions.

Table 4.2: Description of the different BaseSpace applications used in data analysis pipeline and the specific parameters used for each software application.

Analysis Application	Function	Parameters Utilised
FASTQC	Provides simple quality controls checks on raw sequence data and gives a quick insight into any biases or issues with the sequence reads.	• Kmer Size: 5 Kmer
FASTQ Toolkit	Enables FASTQ file manipulations including adapter trimming, quality trimming, length filtering, format conversions and down-sampling.	• Minimum read length: 32 • Adapter trimming • Base trimming-5' end trimmed by 10 positions & 3' end trimmed by 4 positions
RNA-seq Alignment	Maps RNA-seq reads to a reference genome. Spliced Transcripts Alignment to a Reference (STAR) alignment software specifically address the common challenges of RNA-seq alignment including detection of mismatches, insertions and deletions	• Genome-**UCSC (susScr3)** • STAR aligner • Trim TruSeq adapters
Cufflinks Assembly & Differential Expression.	Estimates expression values and calculates differential expression.	• Genome-**UCSC (susScr3)** • Strandedness-First strand

4.3) Results:

4.3.1) Experimental Design Background:

Transcriptome sequencing was applied to identify differentially expressed genes in IPEC-J2 cells between Cd exposed cells and non-exposed cells and to enable the evaluation of the differences in gene expressions between the Se pre-treated samples exposed to Cd. IPEC-J2 cells were subjected to seven different treatment conditions (see Table 4.3). Each treatment condition was carried out in triplicate resulting in a final sample number of twenty-one samples.

Table 4.3: Treatment conditions applied to IPEC-J2 cells.

Treatment Condition	Average Number of Raw Reads per Triplicate
No Se + No Cd	1,059,325
No Se + Cd	718,927
Se-YA + Cd	1,028,576
Se-YB + Cd	1,023,531
Se-YC + Cd	851,684
Se-Ni + Cd	682,797
Se-M + Cd	1,069,694

IPEC-J2 cells were exposed to each of the seven treatment conditions leaving one week in between the cultivation and treatment of the next batch of cells. This process was repeated three times to produce biological replicates. The overall experimental design is illustrated in Fig. 4.3, where numbers indicate experimental day each component was carried out, the first three lines indicate sequential replicates to generate RNA extracts and the last line details the quality testing and sequencing of the extracted RNA.

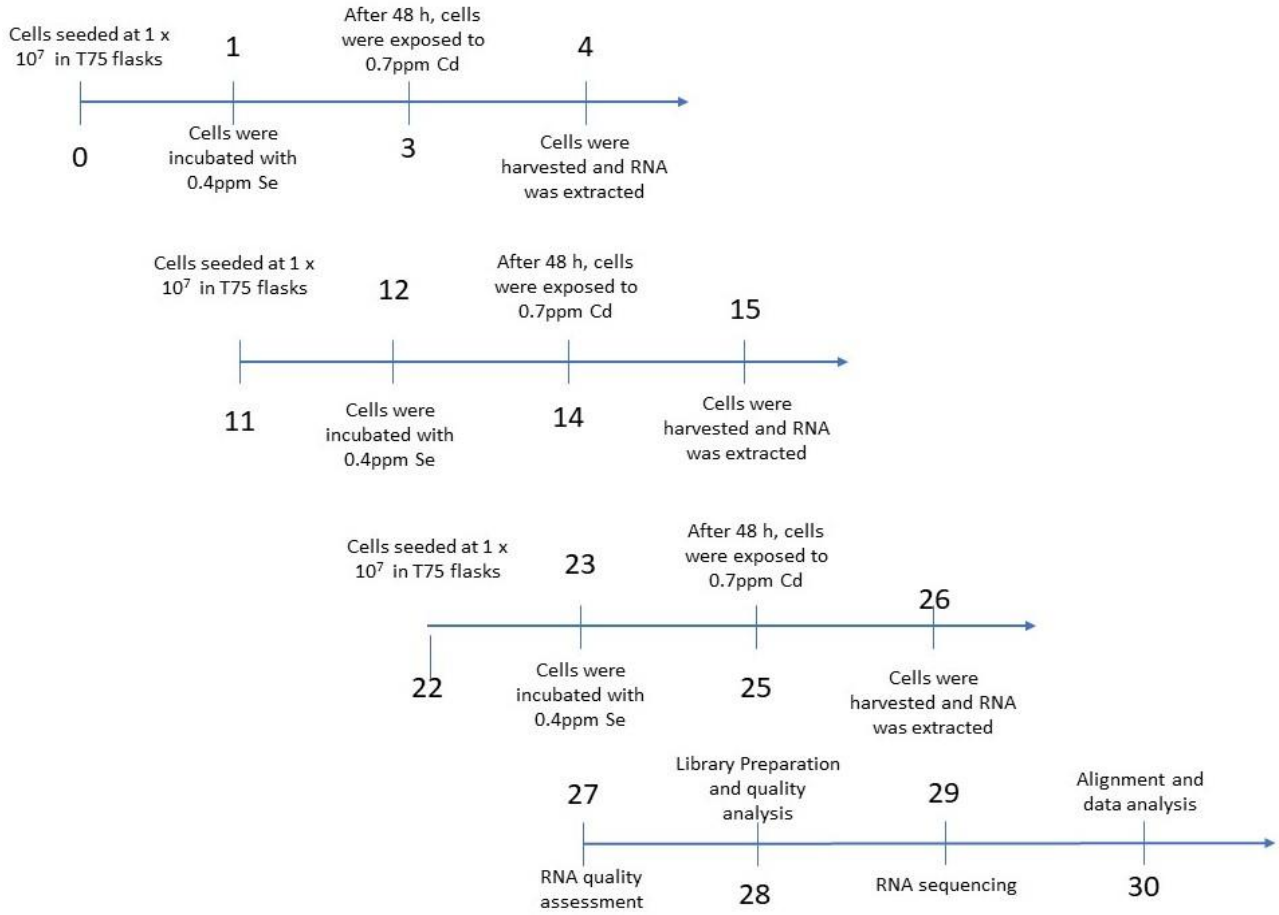


Figure 4.3: Timeline diagram illustrating the experimental design strategy utilised to produce biological replicates and the overall workflow of the RNA-sequencing experiment.

4.3.2) RNA Integrity Analysis:

The integrity of all the twenty-one total RNA samples was assessed using the RNA 6000 Nano Kit and the Agilent 2100 bioanalyzer (Fig. 6.1 – 6.2, appendix). Total RNA is comprised mainly of ribosomal RNA (>80%), with the rRNA species 28S and 18S comprising the majority of the rRNA. The quality of mRNA is assessed by determining the 28S:18S ratio as the method assumes that rRNA quality and quantity reflect that of the mRNA population. Mammalian 28S and 18S are 5kb and 2kb in size respectively, therefore a ratio of 2.1 is regarded as the benchmark of high quality RNA (32). The RNA integrity number (RIN) is an algorithm that defines RNA integrity by analysing important characteristics over several

regions of the recorded electropherogram. The RIN provides a more robust method of RNA quality than using the 28S:18S ratio alone (33). All twenty-one samples analysed returned a RIN number of 10 indicating high quality RNA.

4.3.3) RNA Sequence Alignment:

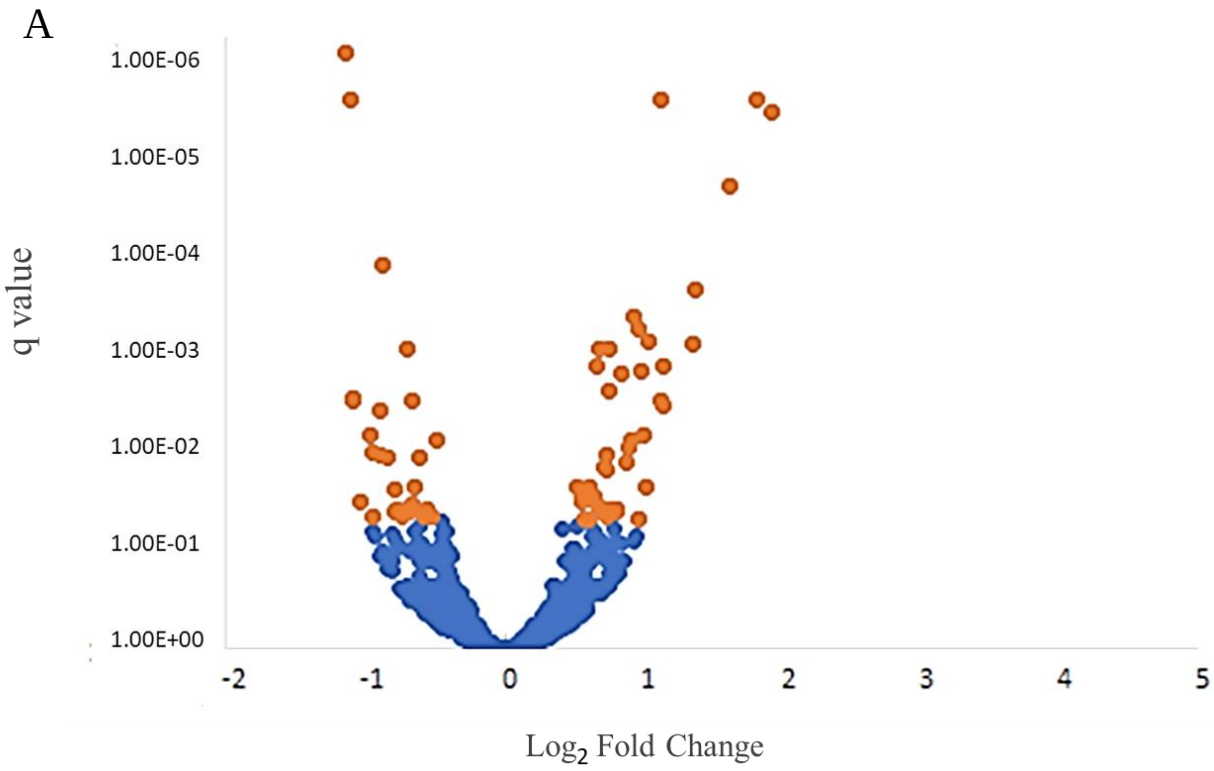
FASTQC quality assessment of RNA-seq reads was performed prior to sequence alignment (Fig. 6.3 – 6.11, Appendix). The RNA-seq data was aligned and compared to the *Sus scrofa* reference genome (Pig) as provided by UCSC (susScr3, Aug. 2011). RNA alignment was performed using Cufflinks Assembly & DE v2.0.0 using the STAR aligner. Normalisation of hits was carried out using Cuffnorm with compatible normalisation used to generate the number of mapped hits. The false discovery rate was set at 0.05. After quality control, approximately between 80.8% to 86.9% of reads were mapped to the reference genome (see Table 4.4).

Table 4.4: Summary of RNA alignment results illustrating the percentage of reads passing the filter that aligned to the reference genome, the percentage of reads that did not align to the genome and the percentage of reads that aligned to abundant transcripts such as mitochondrial and ribosomal sequences.

Sample ID	Read Length	Number of Reads	% Total Aligned	% Abundant	% Unaligned	Median CV Coverage Uniformity	% Stranded
Cd1	1x76	754,632	84.61%	5.87%	15.39%	0.79	99.59%
Cd2	1x76	667,872	84.24%	5.94%	15.76%	0.80	99.63%
Cd3	1x76	734,269	83.85%	5.21%	16.15%	0.79	99.78%
H1	1x76	398,779	86.54%	5.29%	13.46%	0.77	99.40%
H2	1x76	2,027,791	85.56%	5.53%	14.44%	0.65	99.71%
H3	1x76	751,405	83.57%	4.78%	16.43%	0.80	99.31%
Ni1	1x76	716,643	84.13%	5.93%	15.87%	0.81	99.36%
Ni2	1x76	785,941	84.01%	5.95%	15.99%	0.81	99.73%
Ni3	1x76	546,283	85.05%	7.28%	14.95%	0.77	99.33%
SeM1	1x76	1,759,327	83.10%	5.18%	16.90%	0.67	99.75%
SeM2	1x76	740,378	84.41%	6.79%	15.59%	0.82	99.73%
SeM3	1x76	709,379	84.18%	5.91%	15.82%	0.83	98.81%
Sp1	1x76	867,463	86.52%	6.18%	13.48%	0.73	99.52%
Sp2	1x76	838,294	83.80%	6.66%	16.20%	0.79	99.70%
Sp3	1x76	1,381,086	86.93%	6.49%	13.07%	0.65	99.50%
YA1	1x76	681,517	84.42%	5.49%	15.58%	0.81	99.33%
YA2	1x76	714,739	80.84%	6.72%	19.16%	0.82	99.80%
YA3	1x76	1,675,394	87.15%	5.64%	12.85%	0.62	99.33%
YS1	1x76	688,497	84.60%	5.93%	15.40%	0.81	99.78%
YS2	1x76	1,225,536	86.64%	5.74%	13.36%	0.68	99.32%

4.3.4) Visualisation of Differential expression (Volcano Plots):

The differential gene expression data generated by Cufflinks Assembly & DE v2.0.0 was used to plot significantly dysregulated genes versus non-significant genes in volcano graph format. As mentioned above, Cd/No Se versus No Cd/No Se group showed dysregulation of 167 genes (Fig. 4.4A), Cd/No Se versus Cd + Se-YA resulted in dysregulation of 9 genes (Fig. 4.4B), 15 genes were differentially expressed between the Cd/No Se versus Cd + Se-YB treatment (Fig. 4.4C) and 6 genes were dysregulated between the Cd/No Se versus Cd + Se-YC treatment (Fig. 4.4D). There was a significant difference in the gene dysregulation pattern observed between the Cd/No Se plot (Fig. 4.4A) and the Cd and Se-Y treated groups (Fig. 4.4B-D).



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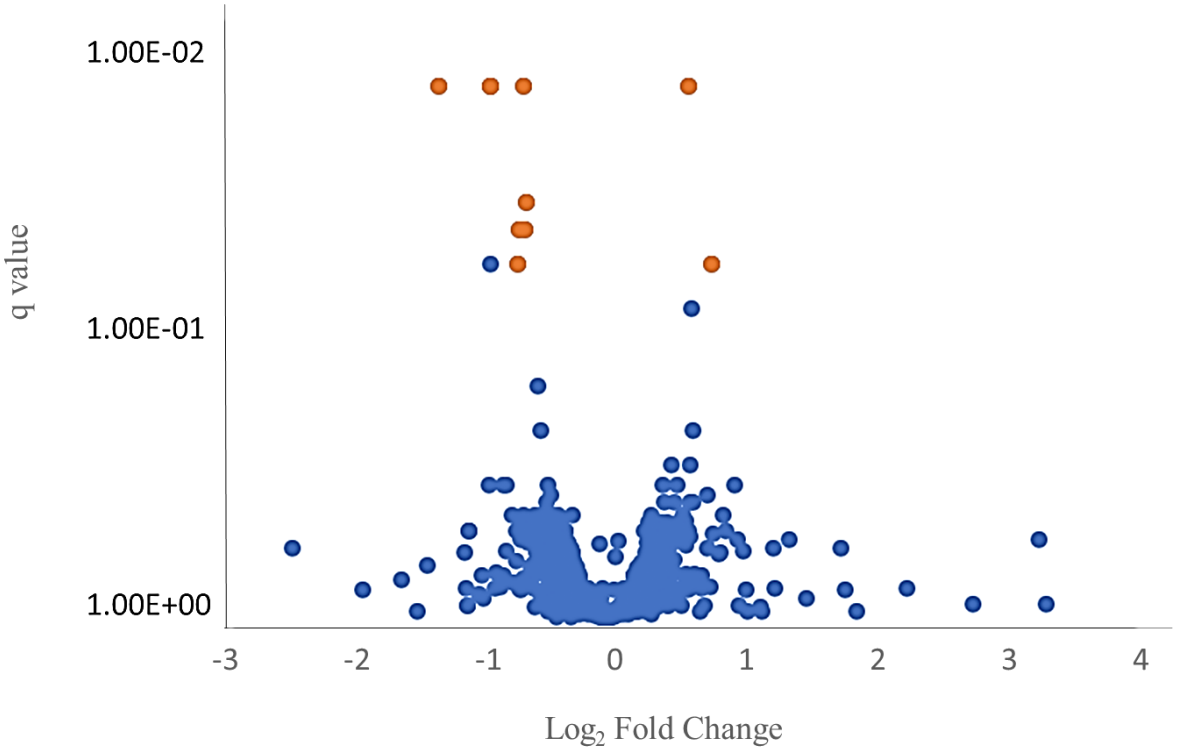
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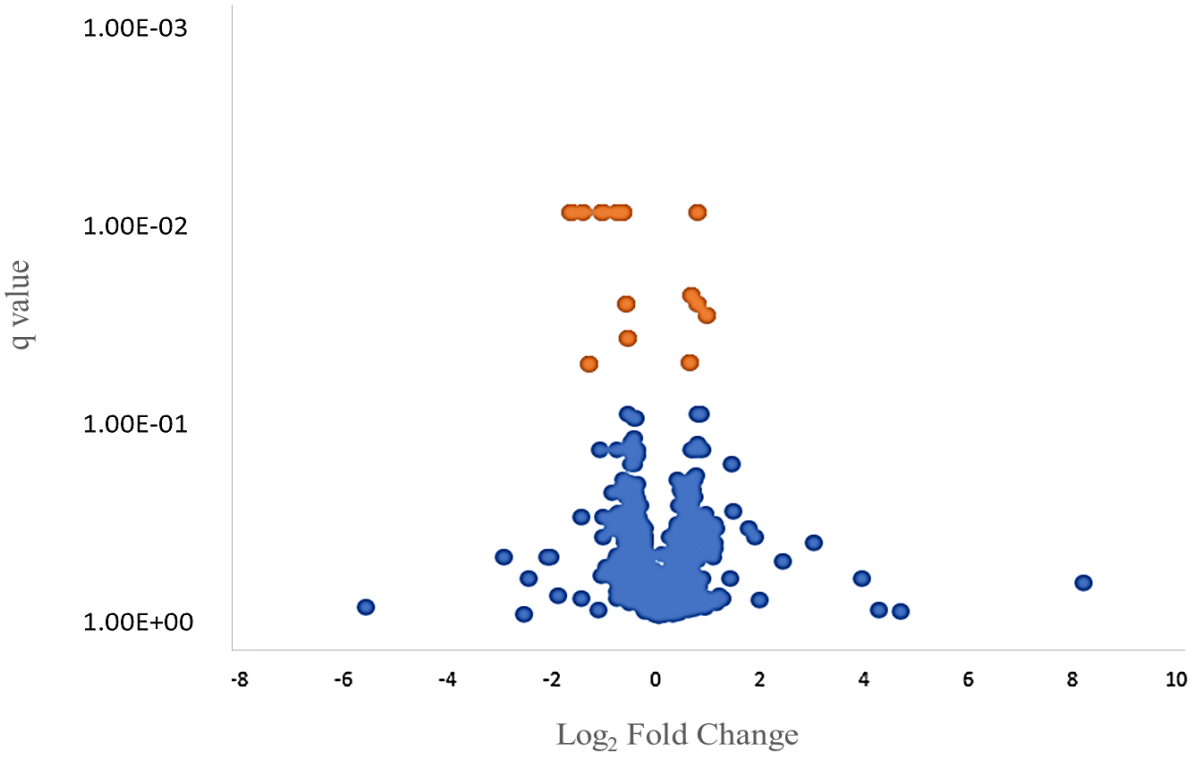
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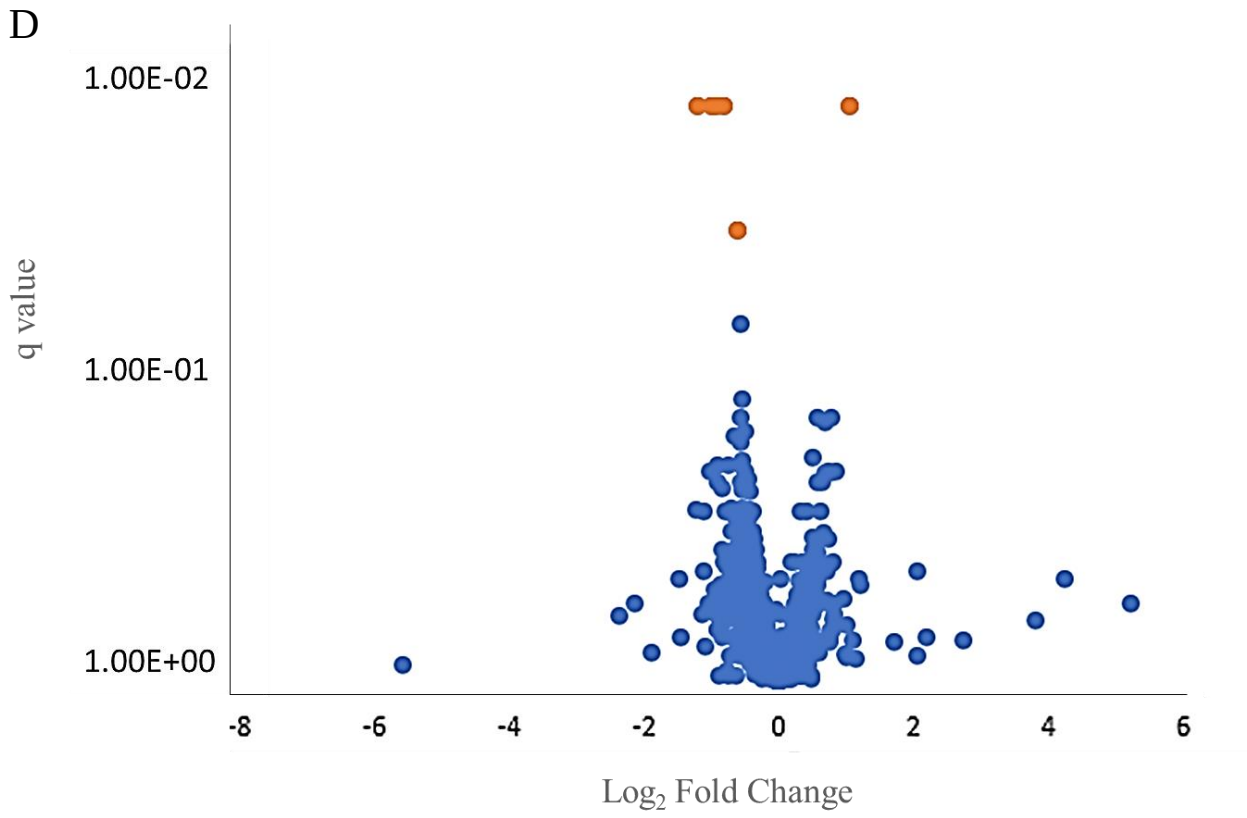


Fig. 4.4: Volcano plots displaying the differentially expressed genes based on RNA-seq data. Blue dots represent non-differentially expressed genes, while orange dots represent significantly differentially expressed genes. The x-axis values correspond to the log_2 fold change value and the y-axis corresponds to the q-value. A) Differentially expressed genes between Cd/No Se and No Cd/No Se treatment, B) Differentially expressed genes between Cd/No Se and Cd + Se-YA supplementation, C) Differentially expressed genes between Cd/No Se and Cd + Se-YB treatment, D) Differentially expressed genes between Cd/No Se and Cd + Se-YC.

4.3.5) Differential Gene Expression Analysis:

Differential gene expression analysis was performed using Cufflinks Assembly & DE v2.0.0. In total between all the groups analysed there were 200 genes dysregulated (See Tables 4.5 – 4.8). There were no differentially expressed genes detected between Cd only treated group and the Se-Ni + Cd/Se-M + Cd treated groups, therefore results from these groups are not represented in this section. Cd exposure was observed to have an extensive effect on gene expression in comparison to No Cd/No Se treated cells (Table 4.5). In total between these two group 167 genes were dysregulated with observations of both upregulation and downregulation with varying effects on fold change. Gene expression profiles of the three different Se-Y compounds are shown in Table 4.6 – 4.8. Firstly, it was observed in all cases that co-exposure with the Se-Y compound altered the profiles of the genes regulated. Secondly, each of the Se-Y compounds had a slightly different profile of gene expression, indicating that each of the Se-Y preparations had a different impact on the cells. All the genes upregulated and downregulated in Cd + Se-YC were also upregulated and downregulated in Cd + Se-YA and Cd + Se-YB, therefore Se-YC gene expression can be viewed as a subset of the gene expression profiles of Se-YA and Se-YB. In addition to the six genes differentially expressed in Se-YC, three more genes were differentially expressed on Se-YA exposure, and 9 additional genes were differentially expressed on Se-YB exposure.

Table 4.5: The top 15 genes differentially expressed between the Cd only group and the No Se/No Cd treated group (according to q value and fold change). A total of 167 genes were dysregulated between these two sample groups (See appendix Table 6.1 page 15).

Gene	Log_2 No Cd/ No Se FPKM)	Log_2 (Cd FPKM)	Log_2 Fold Change
BNP	-10	6.07	-16.07
MT1A	5.93	11.41	-5.48
IL8	3.19	6.7	-3.51
HSPH1	4.72	6.99	-2.27
CLU	3.08	5.3	-2.22
TXNRD1	4.92	6.85	-1.92
DNAJB1	5.71	7.55	-1.84
HSPA8	8.08	9.83	-1.74
ASNS	7.93	6.78	1.14
CD70	8.03	6.87	1.16
HADHA	7.11	5.87	1.24
PCK2	5.57	4.28	1.3
PLIN2	5.92	4.57	1.35
FOS	7.97	6.32	1.65
RENBP	5.3	3.58	1.72

Table 4.6: 9 genes differentially expressed between the Cd only group and the Cd + YA group. Of these 9 genes, 2 genes were upregulated, and 7 genes were downregulated.

Gene	Log_2 (Cd FPKM)	Log_2 (Se-YA FPKM)	Log_2 Fold Change	q Value
DNAJB1	9.39	8.51	-0.89	1.46E-02
HSPH1	8.83	8.2	-0.63	1.46E-02
IL8	8.54	7.25	-1.29	1.46E-02
PHLDA2	12.24	11.35	-0.89	1.46E-02
RN18S	10.4	11.08	0.68	1.46E-02
GADD45G	9.08	8.42	-0.66	4.57E-02
HSP90AA1	12.67	12.07	-0.61	4.57E-02
SEPHS2	8.28	7.67	-0.61	4.57E-02
AD014	9.49	10.33	0.85	4.87E-02

Table 4.7: Tabular representation of the differential expression of genes between the Cd only treated group and the Se-YB treated group. A total of 15 genes were dysregulated, with upregulation of 5 genes and downregulation of 10 genes.

Gene	Log_2 (Cd FPKM)	Log_2 (Se-YB FPKM)	Log_2 Fold Change	q Value
DNAJB1	7.57	6.12	-1.44	8.04E-03
GADD45G	7.25	5.62	-1.63	8.04E-03
HSP90AA1	10.85	10.13	-0.72	8.04E-03
HSPA8	9.84	9.04	-0.8	8.04E-03
HSPH1	7	5.92	-1.08	8.04E-03
IL8	6.71	5.04	-1.68	8.04E-03
MLLT11	5.47	4.4	-1.07	8.04E-03
PHLDA2	10.41	9.74	-0.68	8.04E-03
RN18S	8.58	9.29	0.71	8.04E-03
SLC2A2	6.44	7.05	0.61	2.17E-02
ATP6V1A	5.16	5.87	0.71	2.41E-02
NOP56	7.33	6.7	-0.62	2.41E-02
KDELR3	4.53	5.42	0.9	2.78E-02
RANBP1	7.9	7.31	-0.59	3.62E-02
ASNS	6.8	7.37	0.57	4.83E-02

Table 4.8: Tabular representation of the dysregulation of genes between the Cd only treated group and the Se-YC treated group. The results demonstrated the dysregulation of 6 genes, with 5 genes displaying downregulation and 1 gene being upregulated

Gene	Log_2 (Cd FPKM)	Log_2 (Se-YC FPKM)	Log_2 Fold Change	q Value
DNAJB1	7.58	6.66	-0.92	1.40E-02
GADD45G	7.26	6.06	-1.2	1.40E-02
IL8	6.72	5.73	-1	1.40E-02
PHLDA2	10.42	9.61	-0.82	1.40E-02
RN18S	8.59	9.63	1.04	1.40E-02
RANBP1	7.91	7.3	-0.61	3.51E-02

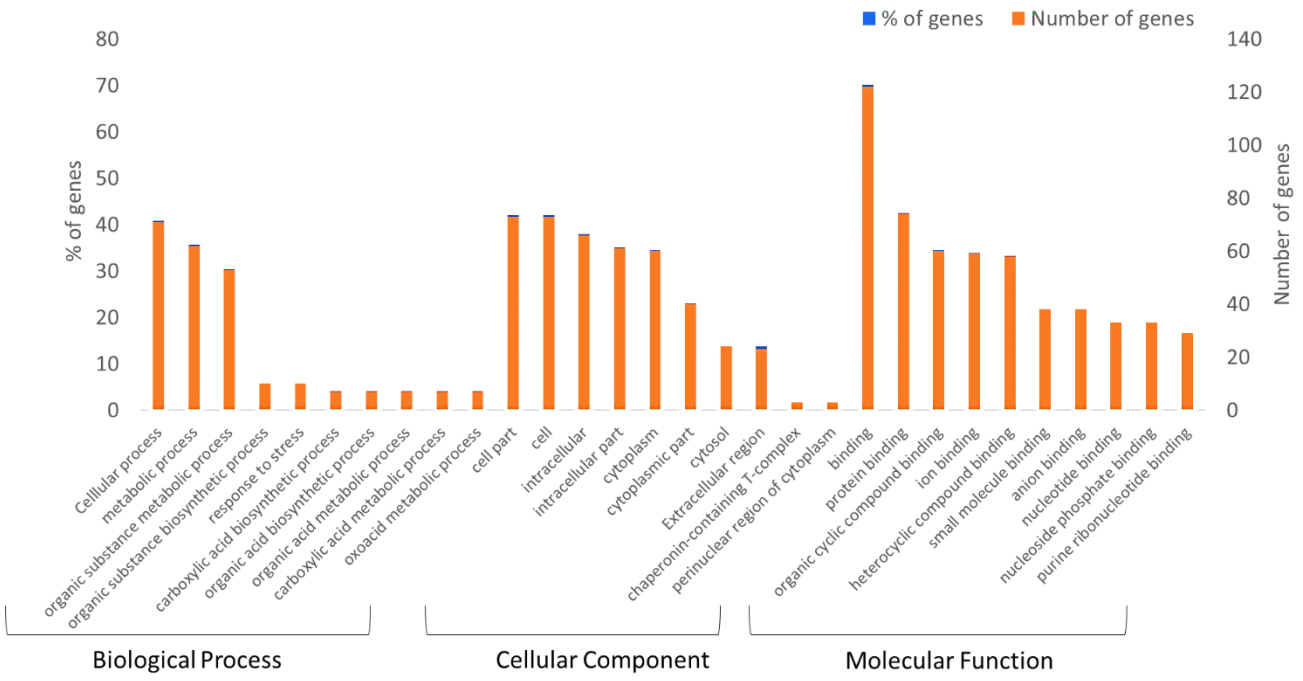
4.3.6) Gene Ontology Enrichment:

Gene Ontology (GO) functional enrichment analysis (http://geneontology.org/) was performed on the statistically significant differentially expressed genes shown in Figures 4.5, A-D, to identify the pathways that may have been influenced by the seven different treatments analysed by RNA seq technology. Fisher's exact test was implemented in the Panther Pathways software (http://pantherdb.org/) to determine whether the proportion of the variables tested in this present study differed with the gene ontology data available. The false discovery rate (FDR) was set at <0.05.

The GO functional enrichment analysis of the Cd/No Se exposed cells showed that within the biological process, the maximum enriched categories as a result of Cd exposure included cellular process (40.8%), metabolic process (35.6%) and organic substance metabolic process (30.4%), shown in Figure 4.5A. Within the cellular component, the maximum enriched gene categories included the cytoplasm (34%) and extracellular region (13.7%). Within molecular function, the maximum enriched gene categories included protein binding (42.5%), organic cyclic compound binding (34.4%) and ion binding (33.9%).

The exposure of Se-yeasts in addition to Cd also resulted in differences in expressed differential gene regulation, shown in Figures 4.5B-D. The GO enrichment analysis illustrated that within the biological process for the Cd + Se-YC analysis the maximum enriched genes included regulation of growth hormone function (66%), biological regulation (83%) and regulation of signal transduction (50%). Within the molecular function class, the maximum enriched gene categories included interleukin-8 receptor binding (16%), CXCR chemokine receptor binding (16%) and chemokine receptor binding (16%). The GO enrichment analysis illustrated that within the biological process for the Cd + Se-YA analysis the maximum enriched genes were response to unfolded protein (44%), response to topologically incorrect protein (44%), regulation of cellular response to heat (33%). Within the molecular function class, the maximum enriched gene categories included protein binding (33%), unfolded protein binding (22%) and chaperone binding (10%). Within the biological process for the Cd + Se-YB analysis the maximum enriched genes included biological regulation of protein insertion (75%), establishment of protein localization (56%) and regulation of signal transduction (31%). Within the molecular function class, the maximum enriched gene categories included purine nucleotide binding (43%), nucleotide binding (43%) and nucleoside phosphate binding (40%).

A


% of genes
Number of genes
% of genes
Number of genes
Cellular process
metabolic process
organic substance metabolic process
organic substance biosynthetic process
response to stress
carboxylic acid biosynthetic process
organic acid biosynthetic process
organic acid metabolic process
carboxylic acid metabolic process
oxoacid metabolic process
cell part
cell
intracellular
intracellular part
cytoplasm
cytoplasmic part
cytosol
Extracellular region
chaperonin-containing T-complex
perinuclear region of cytoplasm
binding
protein binding
organic cyclic compound binding
ion binding
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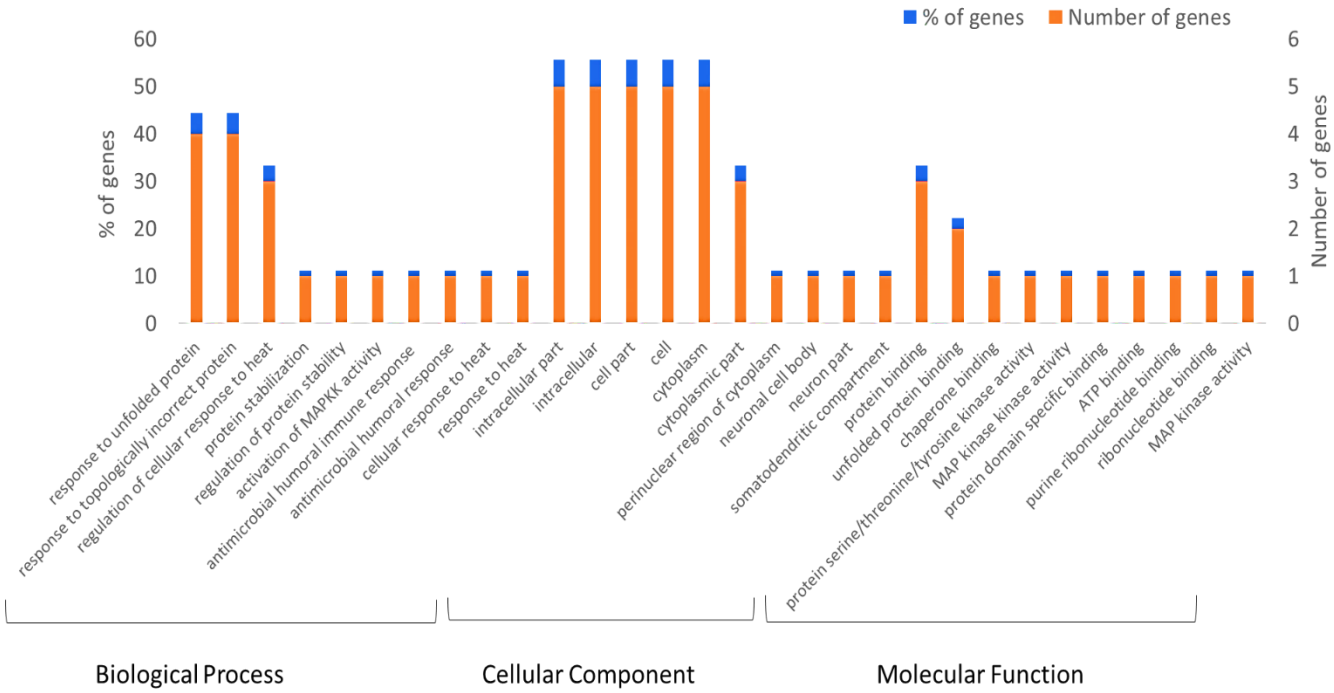
purine ribonucleotide binding
Biological Process
Cellular Component
Molecular Function

B


% of genes
Number of genes
% of genes
Number of genes
response to unfolded protein
response to topologically incorrect protein
regulation of cellular response to heat
protein stabilization
regulation of protein stability
activation of MAPKK activity
antimicrobial humoral immune response
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cellular response to heat
response to heat
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cell
cytoplasm
cytoplasmic part
perinuclear region of cytoplasm
neuronal cell body
neuron part
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chaperone binding
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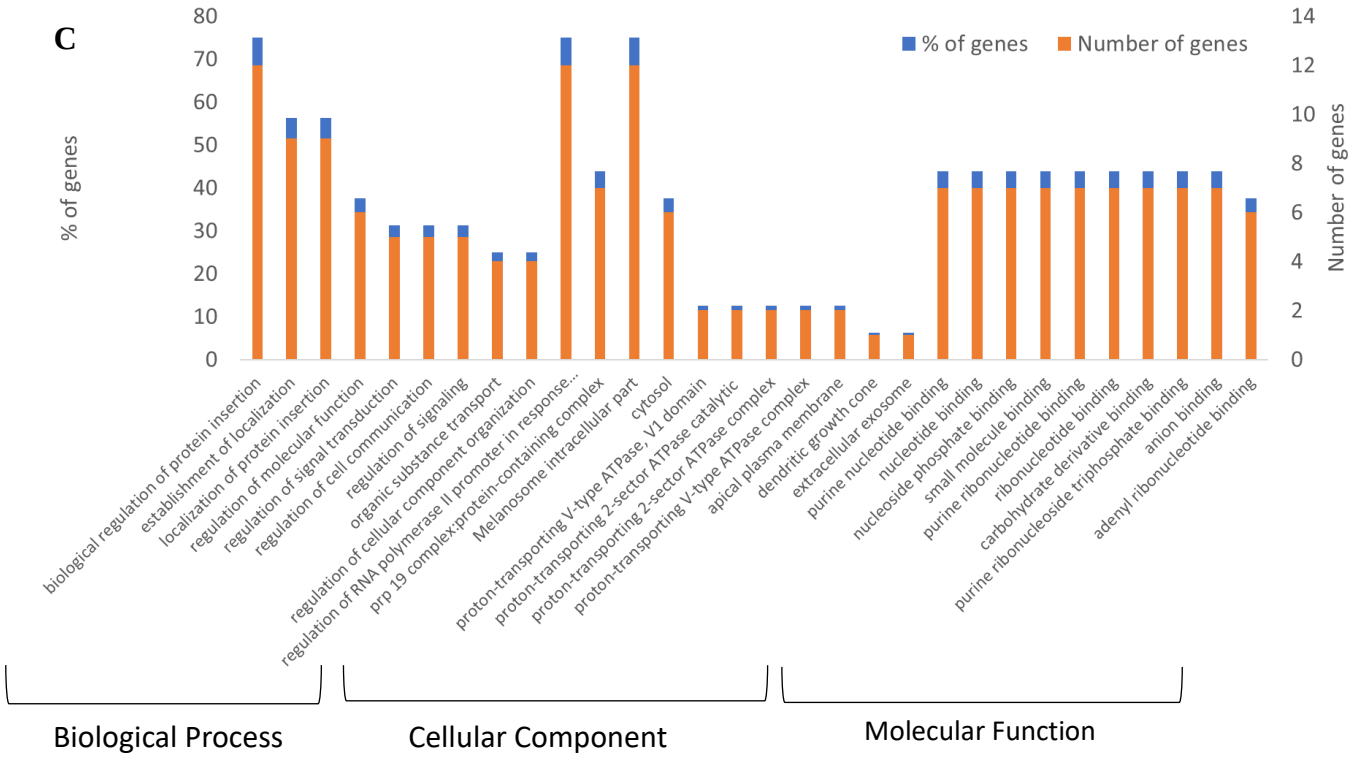
MAP kinase activity
Biological Process
Cellular Component
Molecular Function


C
% of genes
Number of genes
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70
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10
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14
12
10
8
6
4
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biological regulation of protein insertion
establishment of localization
localization of protein insertion
regulation of molecular function
regulation of signal transduction
regulation of cell communication
regulation of signaling
organic substance transport
regulation of cellular component organization
regulation of RNA polymerase II promoter in response...
prp 19 complex:protein-containing complex
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proton-transporting V-type ATPase complex
apical plasma membrane
dendritic growth cone
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adenyl ribonucleotide binding
Biological Process
Cellular Component
Molecular Function

D

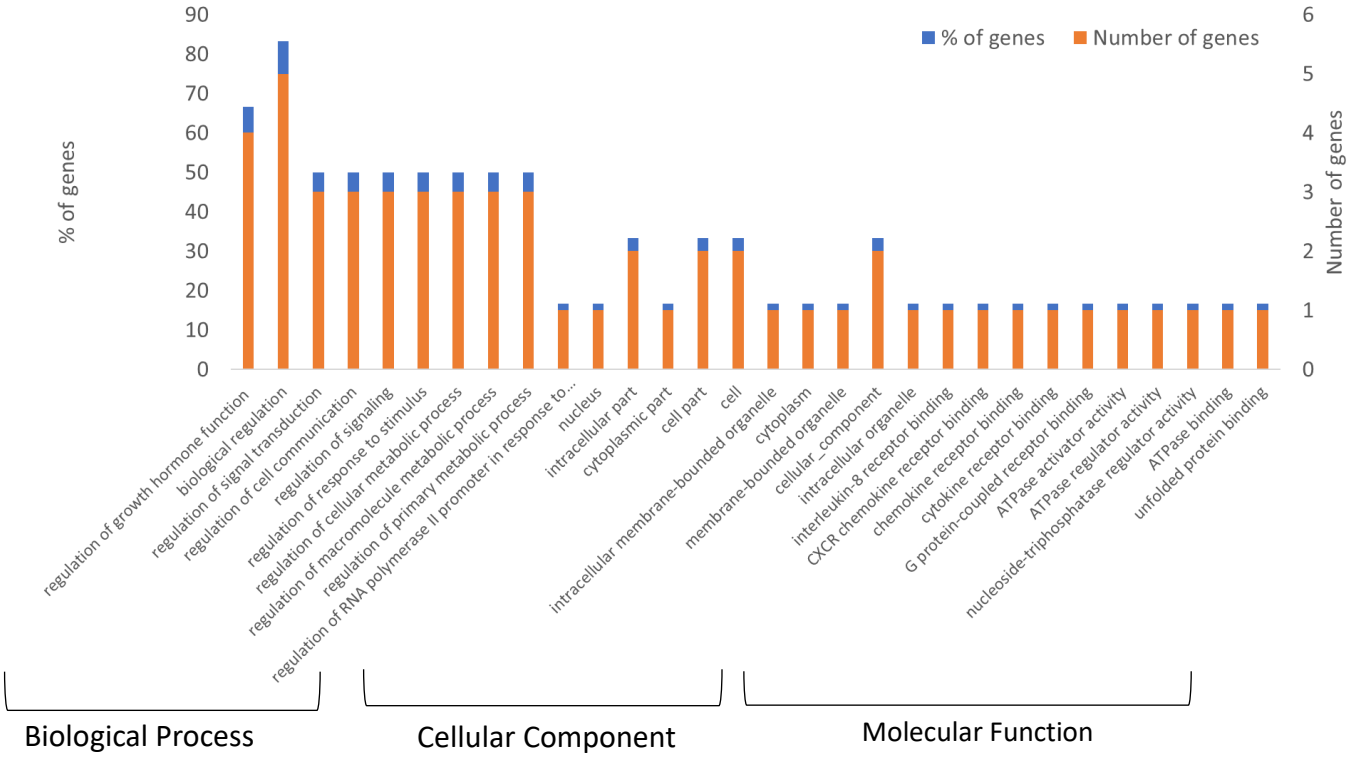

Fig. 4.5: Gene ontology (GO) enrichment analysis A) No Cd/No Se versus Cd/No se treatment, B) Cd/No Se versus Cd + Se-YA supplementation, C) Cd/No Se versus Cd + Se-YB supplementation, D) Cd/No Se versus Cd + Se-YC. The x-axis displays GO terms from the biological process, cellular component and molecular function. While the y-axis shows the number and percentage of gene enrichment.

Discussion:

A significant alteration in gene expression was observed in response to Cd exposure compared to non-exposed cells. Following Cd exposure 167 genes were significantly dysregulated, within this group 28 genes were downregulated and 139 genes were upregulated. The GO functional enrichment analysis was implemented to determine the maximum enriched categories following Cd/No Se exposure. Similar to previous studies that focused on the effects of Cd exposure on gene expression (34–36), organic substance metabolic and biosynthetic process was one of the most enriched categories following Cd/No Se treatment in this present study (Fig. 4.5A). Cd has been shown to induce changes in carbohydrate metabolism and gluconeogenesis. Exposure to Cd resulted in elevated metabolic biomarkers, including increased alanine, glutamate, succinate and glycogen and decreased leucine, β-alanine, hypotaurine and proline (34). Cd has been shown to form several organic amines, sulphur complexes, chloro complexes and chelates, while Cd ions form the soluble salts of arsenates, carbonates and ferrocyanide compounds (35). An enrichment in terms associated with organic substance metabolic and biosynthetic processes which is indicative of the toxicological effects and apoptosis induced by Cd exposure were observed (Fig 4.5) (36). The GO analysis also revealed enrichment of the protein binding and ion binding pathways in response to Cd exposure. Cd exposure resulted in a downregulation of metallothionein (Mt) binding, and reduction in cellular Mt levels has been associated with tumorigenesis (37). Dysregulation of metallothionein can result in defects in protection against heavy metals, oxidative stress, immune reactions and carcinogens in the mouse model (38). A significant enrichment (34 genes) was observed in the response to stress pathway following Cd exposure (Fig. 4.5A), the genes involved in this pathway are involved in stress-activated protein kinase signalling cascade, inflammatory response, intrinsic apoptotic signalling pathway in response to DNA damage and heat shock protein 90. The results indicated that the cells are under substantial

stress following exposure to Cd and upregulation of these pathways may lead to further cellular damage and tumorigenesis. The cellular component enrichment analysis showed signs of cytoplasmic rearrangement and injury. These alterations in cytoplasmic structure has been observed in Cd exposed endothelial cells and has been associated with numerous pathological signs in cell differentiation, heterogeneous chromatin distribution, irregular nuclear envelope and acrosomal dislocation (39, 40).

The addition of Se-Y compounds prior to Cd exposure influenced gene expression patterns in comparison to Cd treatment alone. Differences in GO pathway enrichment analysis between the four treatment groups were observed. GO enrichment analysis determined the maximum enriched gene categories following Cd + Se-YA included response to unfolded proteins, regulation of cellular response to heat and activation of MAPKK activity (Fig. 4.5B). Differential gene expression analysis revealed significant downregulation of the genes in these categories (Fig. 6.13, appendix), indicating that the cells did not experience significant cellular stress when pre-treated with Se-YA and subsequently exposed to Cd. This result is logical as organic Se-Y supplementation has been shown to protect mammalian cells from the damaging effects of oxidative stress (41–43) and has previously been observed by a decrease in cellular stress responses in comparison to Se deficient cells (44). One particular gene that was downregulated following Cd + Se-YA treatment was *SEPHS2* (encoding selenophosphate synthetase 2), evidence shows that this gene is upregulated during Se deficiency and cellular stress (45). The downregulation of *SEPHS2* may suggest a reduction in cellular stress and sufficient Se status following Cd + Se-YA supplementation compared to Cd exposure alone. Se has been shown to dampen the inflammatory response in a murine osteoblast cells model by decreasing the levels of inflammatory molecules and inflammatory related pathways (46). Enrichment of humoral immune response pathways was observed following Cd + Se-YA treatment (Fig. 4.5B), differential gene expression analysis revealed that the genes associated

with these pathways were downregulated (Fig. 6.13, appendix). This may suggest that Se potentially alleviated the inflammatory response to some extent.

The GO functional enrichment analysis of the Cd + Se-YB showed that within the biological process category, the maximum enriched pathway was the regulation of RNA polymerase II in response to stress (Fig. 4.5C). Activation of the RNA polymerase II pathway is an indicator of an organism that is under substantial cellular stress. Oxidative stress induced damage can severely impede RNA polymerase II regulation during transcription and can lead to deleterious lesion and DNA breakage. Non-productive transcription was detected during stress responses in yeast cells by RNA polymerase mapping and significant changes in RNA polymerase II levels was observed during stress response which led to changes in mRNA (47). However, expression of heat shock proteins was downregulated following Cd + Se-YB (Fig. 6.14, appendix) which would suggest a reduction in cellular stress response. Similar to Cd/No Se treatment a downregulation in Mt was observed following Cd + Se-YB (Fig. 6.14) supplementation which may indicate that cellular defence against heavy metals and oxidative stress was decreased.

Similar to Cd + Se-YB and Cd + Se-YB treatment, the GO functional enrichment analysis of the Cd + Se-YC also showed pathway enrichment of the regulation of RNA polymerase II in response to stress suggesting the cells may have been responding to stress (Fig. 4.5D). Within the molecular function category, the cystine-X-cystine chemokine receptors (CXCR) chemokine binding pathway which is associated with inflammatory response was modulated. The various biological effects of IL-8 expression are mediated through CXCRs, it serves as a defence mechanism against cell injury (48). The genes associated with this pathway were downregulated when compared to Cd/No Se treatment suggesting that following Cd +Se-YC treatment the cells were not exposed to significant cellular stress and therefore did not require activation of this pathway. Another pathway that displayed enrichment following GO analysis

in the Cd + Se-YC group was the regulation of response to stimulus (Fig. 4.5D). This result is logical as it indicates that the cells are responding to stimuli or stresses associated with Cd exposure. The genes correlated with this pathway exhibited upregulation following Cd/No Se exposure and displayed downregulation after treatment with Cd + Se-YC suggesting that the cells are less stressed when subjected to pre-treatment with Se-Y prior to Cd exposure.

Conclusion:

Gene expression analysis was utilised to detect potential differential gene expression between a range of Se sources to determine their effect on Cd-induced toxicity and to elucidate the possible genetic mechanisms of Se in response to Cd-induced damage. The RNA seq data revealed a significant modification in gene expression profiles between IPEC-J2 cells exposed to Cd-only treatment and non-exposed cells. The immediate subsequent step in this present study would be RT qPCR analysis for both quality control and authentication of the RNA seq data. In summary, a significant enrichment was observed in the stress response pathways following Cd exposure, indicating that the cells are under substantial stress following exposure to Cd and upregulation of these pathways may lead to further cellular damage. The results highlighted the differences in gene expression between Cd/No Se treated cells and Cd + Se-Y treated samples. A downregulation in stress response and inflammatory response genes was observed following Cd + Se-YA and Cd + Se-YC pre-treatments, suggesting that Se-YA and Se-YC provided the highest level of protection against Cd-induced cellular stress. Supplementation of IPEC-J2 with Cd + Se-YB resulted in upregulation of biomarkers associated with cellular stress and downregulation of the Cd binding Mt protein was observed. The results demonstrated that Se-YB supplementation displayed the lowest capability for the protection against Cd-induced cell injury. This work highlighted that Cd exposure alone

significantly increases cellular stress response pathways in comparison to non-exposed cells. It was demonstrated that organic Se (SeM) and Se-Y compounds differ in their ability to protect the cell from the harmful effects of Cd induced damage. Lastly, variation in the ability of different commercially available Se-Y preparations to protect porcine gut epithelial cells from Cd-induced damage was illustrated. Further research is required to advance the understanding of the role of the yeast component of Se-Y compounds in ameliorating or enhancing heavy metal induced damage.

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Chapter Five: Conclusions and Future Work

Cd contamination of animal feed has become a major problem worldwide and is of concern due to its cytotoxic effect and the potential bioaccumulation of Cd through the food chain (1). Selenium (Se) is an essential trace element that possesses antioxidant properties and can protect the cell against the harmful effects of ROS (2). This thesis investigated the impact of the chemical form of Se on its associated ameliorative effects on Cd induced DNA damage in a porcine model. Se antioxidant effects were shown to be both composition- and dose-dependent. The organic compounds Se-Y and Se-M afforded protection against Cd-induced DNA damage in the IPEC-J2 model system, whereas no protective effects were observed with inorganic Se-Ni and Se-Na supplementation and moreover displayed the ability to enhance the negative effects of Cd-induced DNA damage. The results presented in this study have also highlighted that different commercially available Se-Y preparations vary in their ability to enhance a range of cellular mechanisms that protect porcine intestinal epithelial cells from Cd induced cell injury. Increased bioavailability of Se-Y means a reduction in the quantities of Se required to supplement animal feeds and a decrease in the level of unabsorbed Se excreted into the environment. Therefore, Se-Y supplementation has economic and environmental advantages over traditional inorganic Se sources. Future work is required to further elucidate the role and mechanism of Se-Y in response to heavy metal induced damage.

The transcriptome is the complete set of transcripts in a cell, and relative quantitation of their level under different conditions, as is the case in the present study. RNA-seq gene expression measurements cover a broad dynamic range and can analyse gene expression of a large number of genes (3). Following RNA-seq analysis and the identification of relevant genes of interest, RT qPCR analysis is advisable for both quality and control confirmatory purposes (4). For these reasons, RT qPCR should be performed on selected genes of interest determined by GO functional enrichment analysis in the subsequent stage of this project. The following pathways

that exhibited significant differential expression; stress response, response to unfolded proteins, selenoprotein biosynthesis and RNA polymerase II pathway are of greatest interest.

The results presented in this study (Chapter 3) confirmed the ability of different Se-Y preparations to enhance a range of cellular mechanisms that protected IPEC-J2 cells from Cd-induced damage. The protective effects were concentration-dependent and highlighted the difference in bioefficiency of different Se-Y compounds. Likewise, the RNA-seq differential gene expression analysis also emphasized the difference in bio-efficacy of the three Se-Y compounds. Evidence suggests that different protein-bound Se compounds present in each distinctive Se-Y preparation may induce varying biological effects (5). Therefore, future analyses should include speciation studies using ICP-MS to identify and quantify organic Se species and Se peptides present in the different Se-Y compounds. This future research may shed light on the observed disparity in the biological activities of the different yeast compounds.

The RNA-seq data revealed significant changes in gene expression in response to Cd treatment alone and Se + Cd exposure. The levels of expression of specific proteins of interest including SEPHS2, HSP90, GADD45G, DNAJB1) should also be determined by Western blotting, subject to the availability of suitable antibody reagents. Proteomic studies could also be used to generate broader protein expression profiles from those genes highlighted from the RNA-seq data to be most impacted by Se sources. This approach would also highlight posttranslational changes including ubiquitylation and SUMOylation not revealed by RNAseq as mRNA protein levels from the same gene do not always correlate (6). Two-dimensional differential gel electrophoresis (2-DE) coupled with mass spectrometry analysis can be used to determine the expression levels of selected proteins. whose levels are modulated following various Cd/Se treatments A bespoke protein microarray could also be utilised to generate a protein profile, an approach that has previously been implemented to study the effects of

environmental stresses. However, protein microarrays are costly and controls must be chosen carefully (6, 7).

To further investigate the increased BER activity observed in the *in vitro* DNA repair assay following Se-YA + Cd treatment (8) additional DNA repair assays could be performed. Base excision repair can proceed by two alternative pathways: a DNA polymerase beta-dependent pathway and a proliferating cell nuclear antigen (PCNA)-dependent pathway. These two pathways should be investigated to determine if Se-Y exploits either of these pathways in response to Cd insult. Evidence has shown the occurrence of upregulation of BER utilizing the PCNA binding assay following organic Se treatment (9). Detection of DNA repair factors using immunoblotting could be undertaken in future studies on the effect of Se-Y on Cd-induced DNA damage. Western blotting can be implemented to detect changes in DNA repair factors due to Cd exposure by using the primary antibodies; Rad51, BRCA1 and caspase-7 among others to visualize the proteins (10). Monoclonal antibodies specific to cleaved-PARP1 a DNA repair enzyme that is cleaved by activated caspases or H2AX which is recruited to sites of double-strand DNA breaks could also be used in the elucidation of the DNA repair mechanism by Se-Y compounds. It would also be of benefit to investigate the activity of the Ku protein in response to Cd exposure and Se treatment prior to Cd insult. It has been reported that "*The DSB repair pathway is reliant on the Ku protein and is the primary DNA DSB repair mechanism in mammalian cells"* (11). The Ku protein has also been associated with cell survival and given the RNA-seq results which showed a resistance to cellular stress in Se-YA treated cells this provides support for performing the assay.

Currently, there is a limited number of techniques to determine the DNA repair capacity of cell extracts. The methods presently available include the flow-cytometric host cell reactivation assay (FM-HCR) and two novel assays; the DNA repair molecular beacon (DRMB) assay and the BER oligonucleotide retrieval assay (BER ORA). The FM-HCR assay is a multicolor

fluorescence assay which introduces a defined DNA reporter plasmid containing a specific DNA lesion into a target cell population (12). However, the assay is limited as any alteration in protein production levels unconnected to BER may modify the results (13). The DRMB assay is a fluorogenic plate-based DNA repair assay and allows for the identification and quantification of DNA repair enzymes, DNA repair inhibitors and DNA damage lesions (14). The molecular beacon (a single stranded oligonucleotide probe) contains a 5' fluorophore and a 3' quencher moiety, in the absence of DNA lesions the fluorescence of the fluorophore is quenched while binding of the substrate to the target results in increased fluorescence (15). The BER ORA assay allows for the determination of DNA repair capacity through the transfection of the cell with a DNA lesion, extraction of the DNA following incubation and detection of repair capability using qPCR. The principle of this method relies on the assumption that PCR polymerase is inhibited by the DNA lesion, therefore if the lesion has not been repaired PCR amplification is less efficient across this site. PCR amplification of the test samples relative to the control samples (no DNA damage) will determine DNA repair capacity (13).

The important issue of Cd contamination of animal feed and feed supplements has been emphasised throughout this work. Cadmium (Cd) contamination of animal feed is a major problem worldwide and the bioaccumulation of Cd through the food chain is of grave concern. Contamination of animal feed, feed supplements and water sources with this heavy metal has negative consequences for animal health and also that of the human consumer, being linked with pathologies including neurodegenerative disorders and cancer. Overall this study exhibited the significant potential for the use of Se-Y in animal feed products for protection of gut integrity and protection against Cd induced oxidative DNA damage. The negative implications of supplementation of animal feed with inorganic Se sources have been strongly highlighted through this research. The results of this study are of relevance to the Agri-Food

sector and emphasise the potential economic and environmental advantages of Se-Y over the ubiquitously used inorganic forms.

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15. Maksimenko A, Ishchenko AA, Sanz G, Laval J, Elder RH, Saparbaev MK (2004) A molecular beacon assay for measuring base excision repair activities. *Biochemical and Biophysical Research Communications* 319:240–246 . doi: 10.1016/j.bbrc.2004.04.179

Chapter 6: Appendix

6.1) RNA Integrity Analysis

The electropherograms displayed below (Fig. 6.1 &6.2) shows two well defined peaks at 18S and 28S and does not contain evidence of any degradation or contamination peaks. All twenty-one samples exhibited a 28S:18S ratio of 2.0 and a RIN of 10 which is indicative of high-quality RNA. The bioanalyzer gel image (Fig. 6.2) contains two prominent bands representing 18S and 28S rRNA. The lower marker bands can also be seen at 25 nucleotides (nt), the lower marker is used for alignment. The sharpness of the bands and the absence of low molecular weight smearing signifies high quality, intact total RNA which was observed in all twenty-one samples.

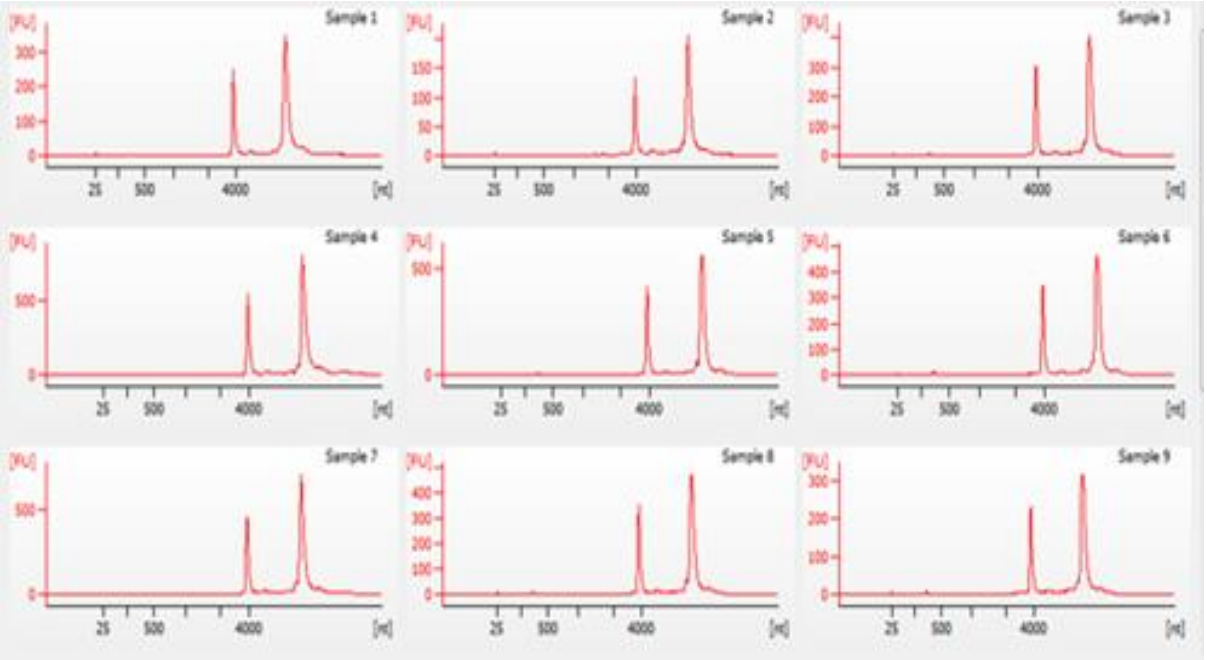

Figure 6.1: Electropherograms displaying intact, high quality RNA samples (RIN 10) and a 28S:18S ratio of 2.0 for all samples.

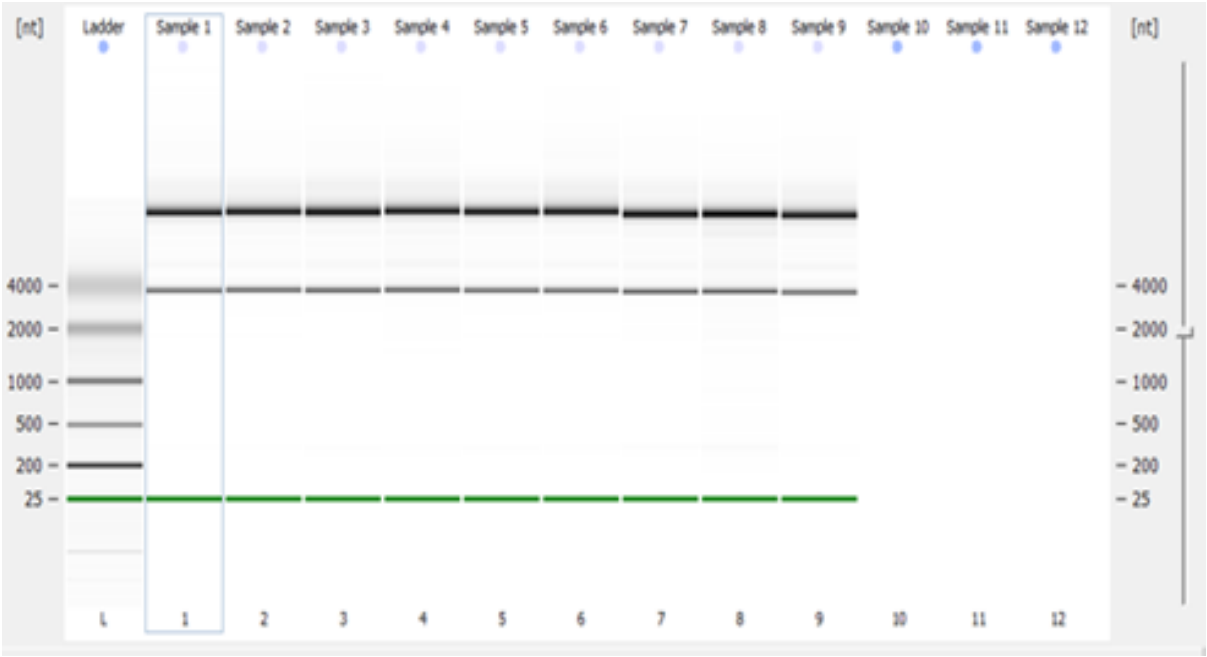

Figure 6.2: Electrophoretic gel image showing a 2:1 ratio between the ribosomal RNAs 28S and 18S.

6.2) FASTQC quality assessment of RNA-seq Reads:

The results displayed in Fig. 6.3 to 6.11 is one example of the quality read results out of the twenty-one samples. The sample result shown below was Se-YC (batch 3) and was chosen at random as a representative example. The quality scores are represented on the Y axis for the Per Base Sequence Quality graph result. The higher the score the better the base call. The background of the plot divides the Y axis into high quality calls (green), intermediate quality calls (orange) and poor-quality calls (red). The results shown in Fig. 6.3 indicate high quality data. The Per Sequence quality Scores plot analyses a subset of the sequences and allows for the detection of universally low-quality values. A warning is raised by the software if the most frequently observed quality score (Phred Score) is below 27. The result illustrated in Fig. 6.4 is indicative of high quality data with a Phred score of >30. Per Base Sequence Content (Fig. 6.5) analysis detects biases between the different bases in a sequence run. A warning is issued if the difference between A and T, C and G is greater than 10%. The lines in this plot are

running in parallel with one another signifying no bias between the bases and a high-quality random library. Fig. 6.6 is a graphical representation of the GC content of each base position in a sequence run. The straight line represented in the graph, illustrates no bias or overrepresented sequences and signifies high quality data. Per sequence GC content (Fig 6.7) analyses GC content across the whole length of each sequence in a run (red) and compares it to a modelled normal distribution of GC content (blue). The central peak of the curve should correspond to the overall GC content of the underlying genome in a normal random library. The result shown in Fig. 6.7 follows the normal shaped distribution of the reference genome and suggests the data is free from bias and/or contamination. N base calls arise when the sequencer cannot make a definitive base call with adequate certainty and substitutes with an N base call instead of a regular base. Fig. 6.8 represents 0% per base N content indicating high quality data. Sequence fragments of uniform length are produced by some high throughput sequencers however, most sequencing instruments generated fragments of varying lengths. The sequence lengths displayed in Fig. 6.9 vary in length from 61-65 bp. It is common to see high duplicate sequence levels in RNA-sequence data because the inherent nature of RNA-sequencing is to count the depth of coverage of each gene. A high increase in the final category (10+) may suggest some type of enrichment bias. The result shown in Fig. 6.10 demonstrates that the values for duplicate levels above 1 quickly decay to zero and there is no significant increase in the final category of the graph. The Kmer content module (Fig. 6.11) determines the enrichment of every 5-mer within the sequence. It works on the assumption that any small fragment of sequence should not have a positional bias in its appearance within a diverse library. In the result displayed below enrichment is observed at the beginning and towards the end of the sequence reads. This is due to the presence of TruSeq adaptors and use of random primers during preparation, however these are later filtered out using the RNA-seq alignment tool. Kmer enrichment can also arise for biological reasons for example the upregulation of

certain sequences. Polyg tails frequently arise in the human genome (with the pig genome being 98% similar to the human genome) and also may arise as a consequence of DNA damage, this may explain the enrichment of polyg tails observed in this graph. In conclusion, all twenty-one data files were determined to be of high quality and suitable for further analysis.

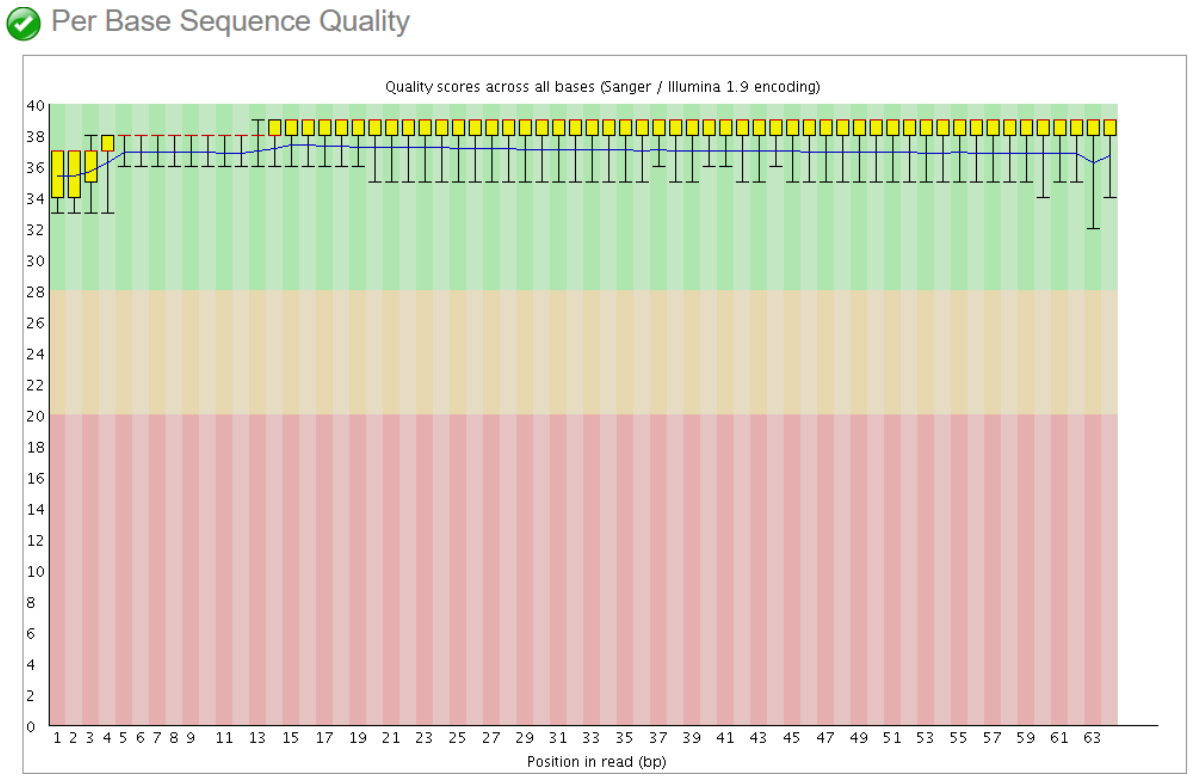

Figure 6.3: FASTQC graph displaying a summary of the range of quality values across all bases.

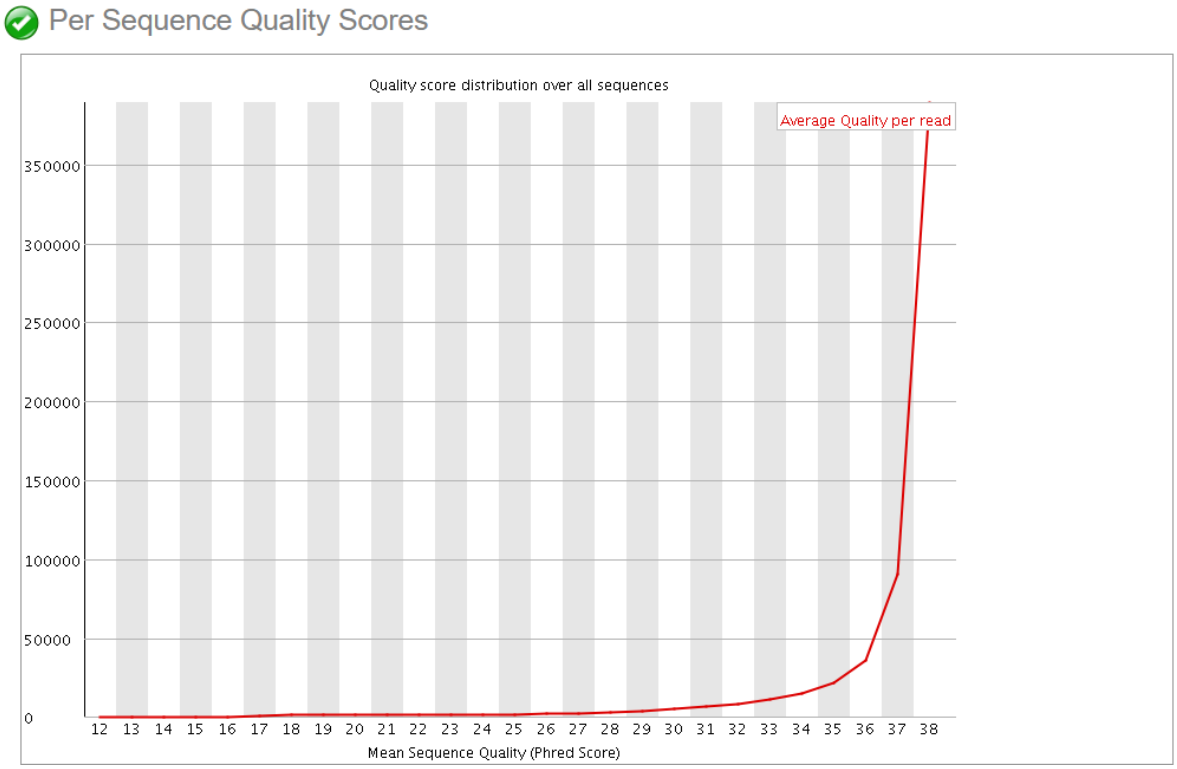

Figure 6.4: Graphical result showing a Phred score of > 30, indicating high quality sequence data.

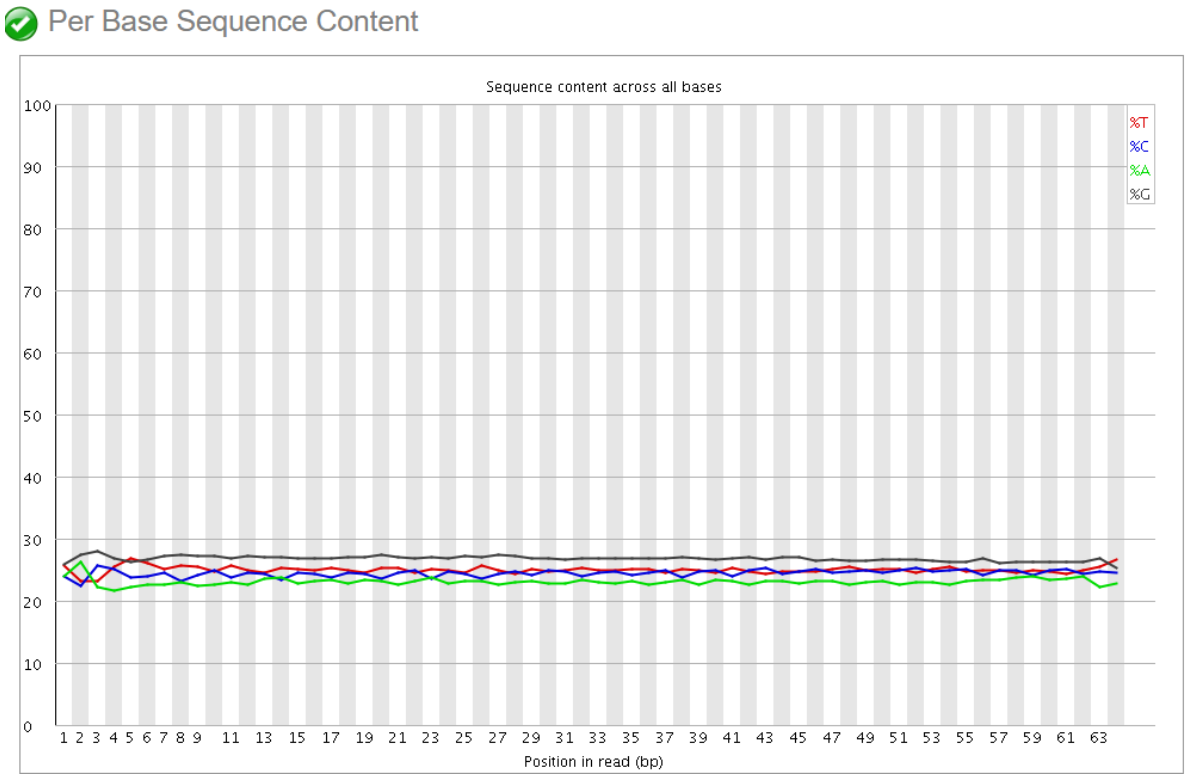

Figure 6.5: Graphical representation of the proportion of each base position in a sequence for which each of the four normal DNA bases has been called. Y axis indicates percentage.

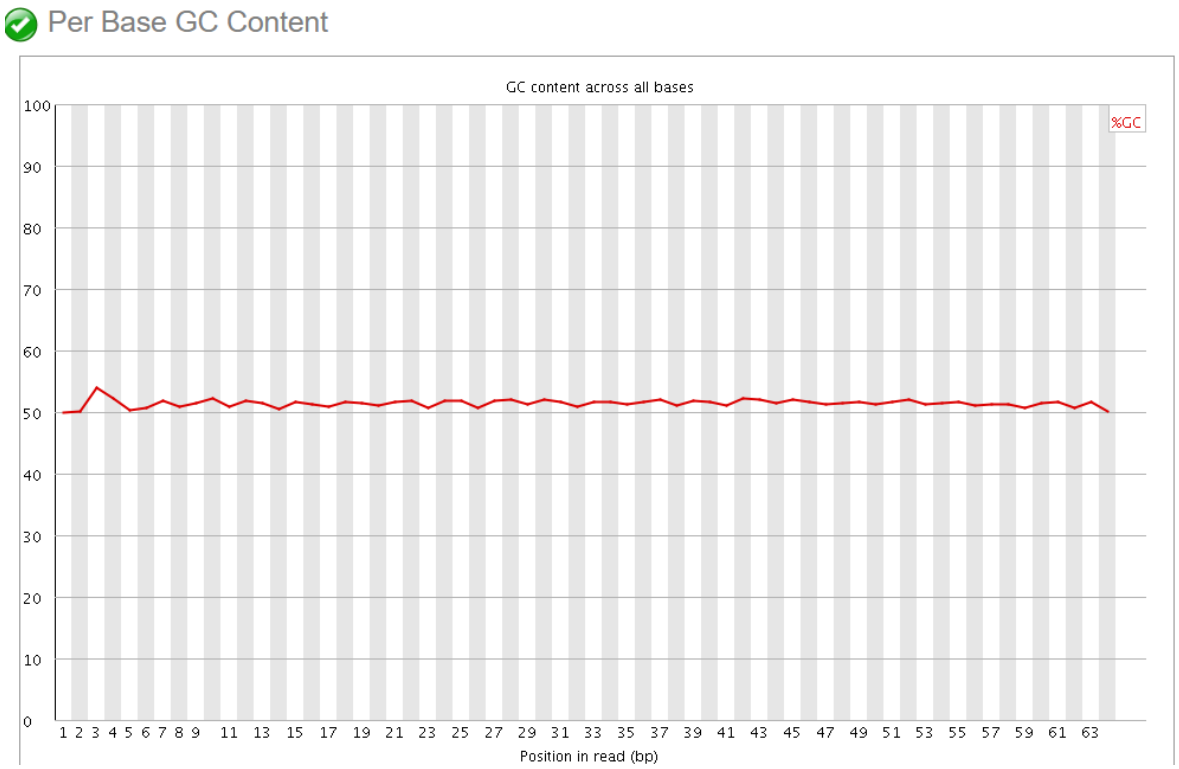

Figure 6.6: FASTQC output portraying the GC content of each base position in a sequence run. Y axis indicates percentage.

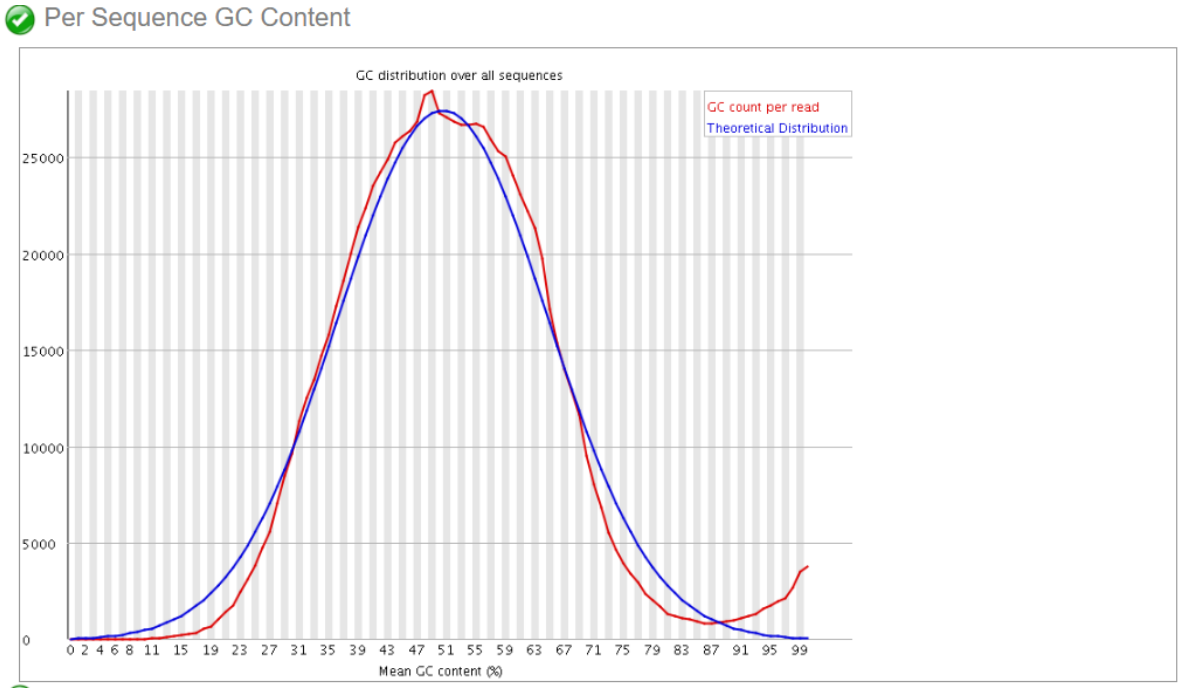

Figure 6.7: Graphical representation of GC content across the whole length of each sequence in a run (red) and compares it to a modelled normal distribution of GC content (blue).

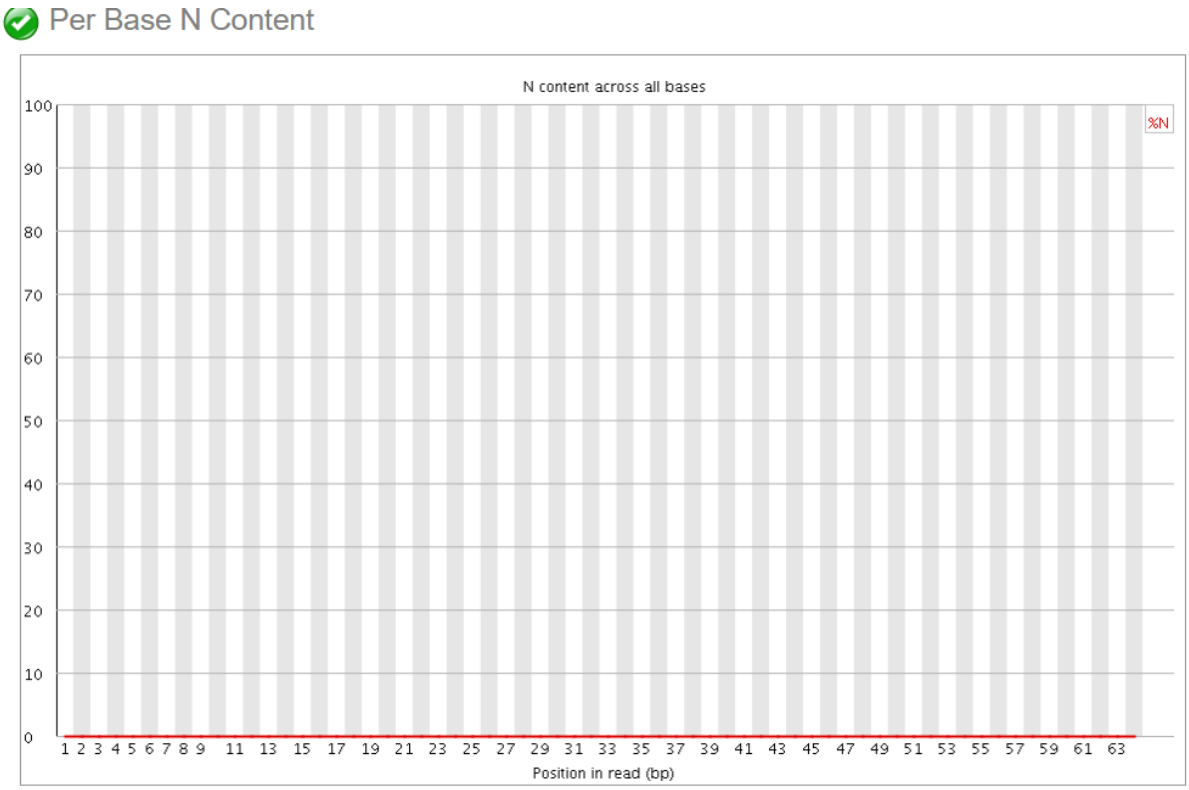

Figure 6.8: Graphical depiction of the number of N base calls that arose during sequencing. Y axis indicates percentage.

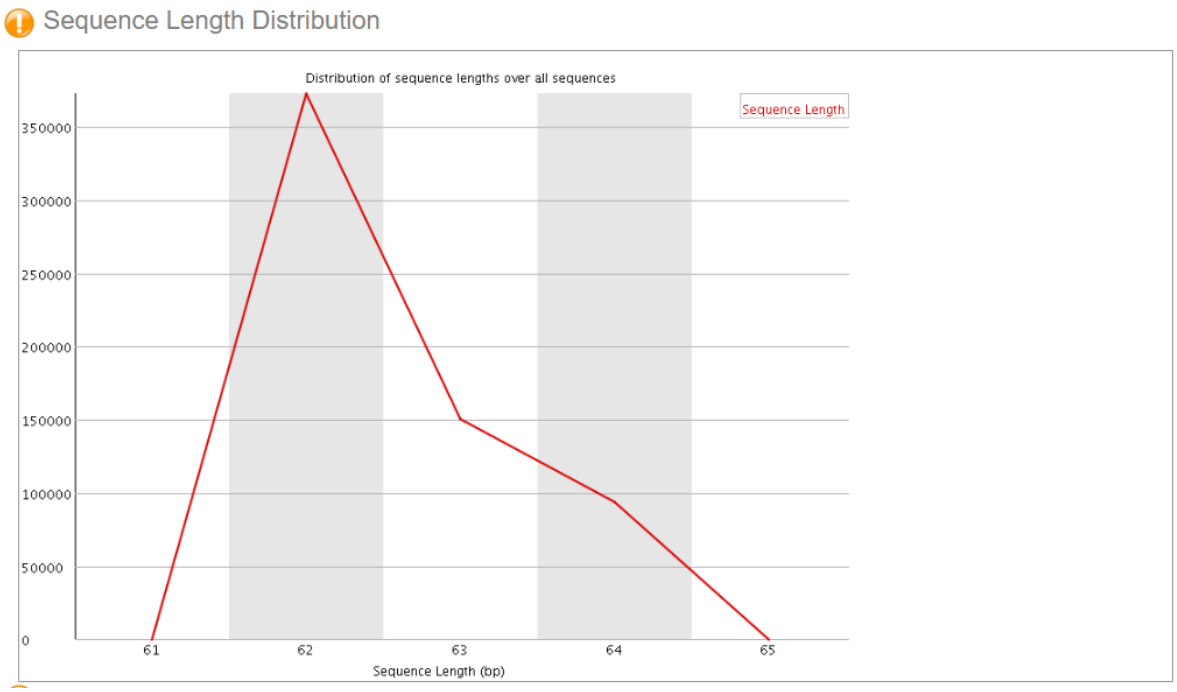

Figure 6.9: FASTQC depiction the distribution of fragment lengths over all sequences.

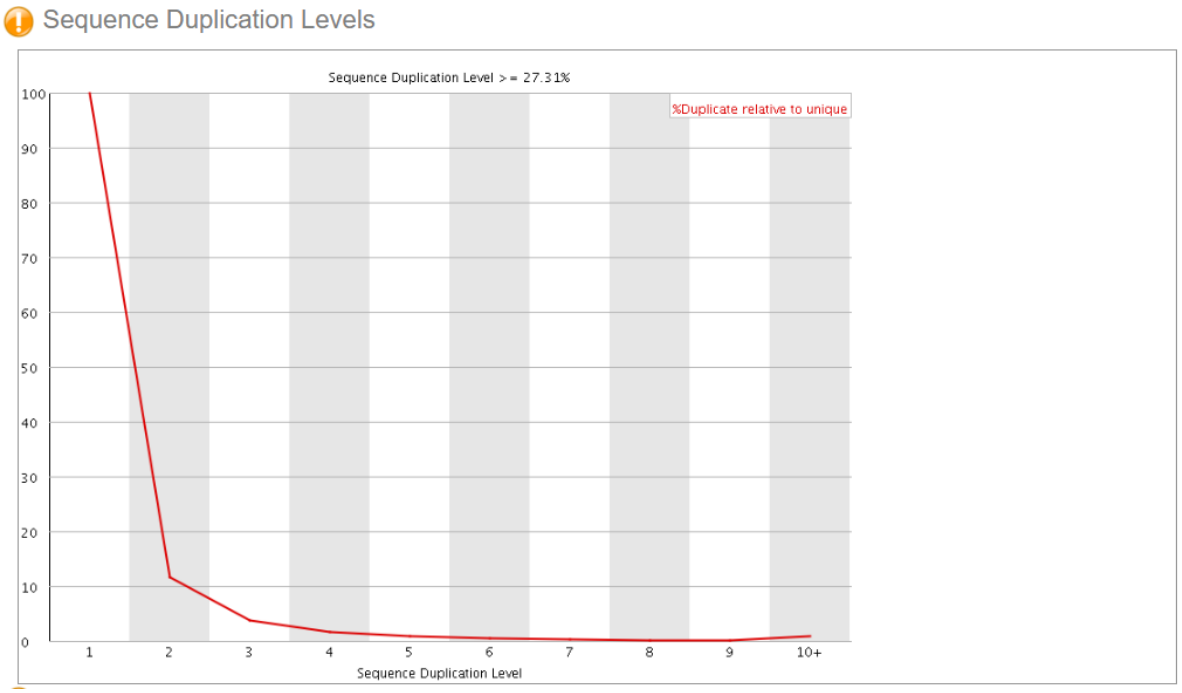

Figure 6.10: FASTQC software output displaying sequence duplication levels.

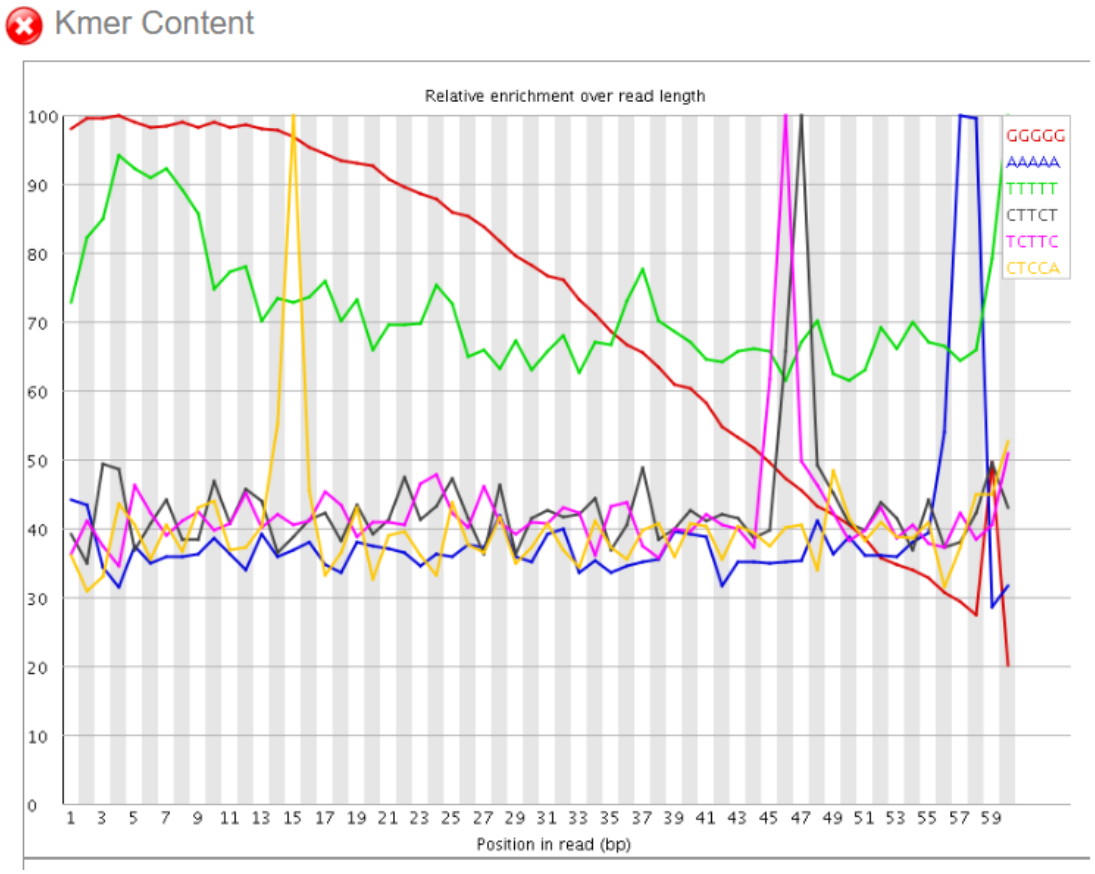

Figure 6.11: FASTQC plot displaying relative Kmer enrichment over read length.

6.3) Differential Gene Expression Heatmaps

The RNA seq data was aligned and compared to the Sus scrofa reference genome (Pig) as provided by UCSC (susScr3, Aug. 2011). Fold change values were obtained using Cuffdiff2 differential gene expression software, the two-way hierarchical clustered heat maps displaying the differential gene expression profiles for each treatment were generated using Cufflinks software.

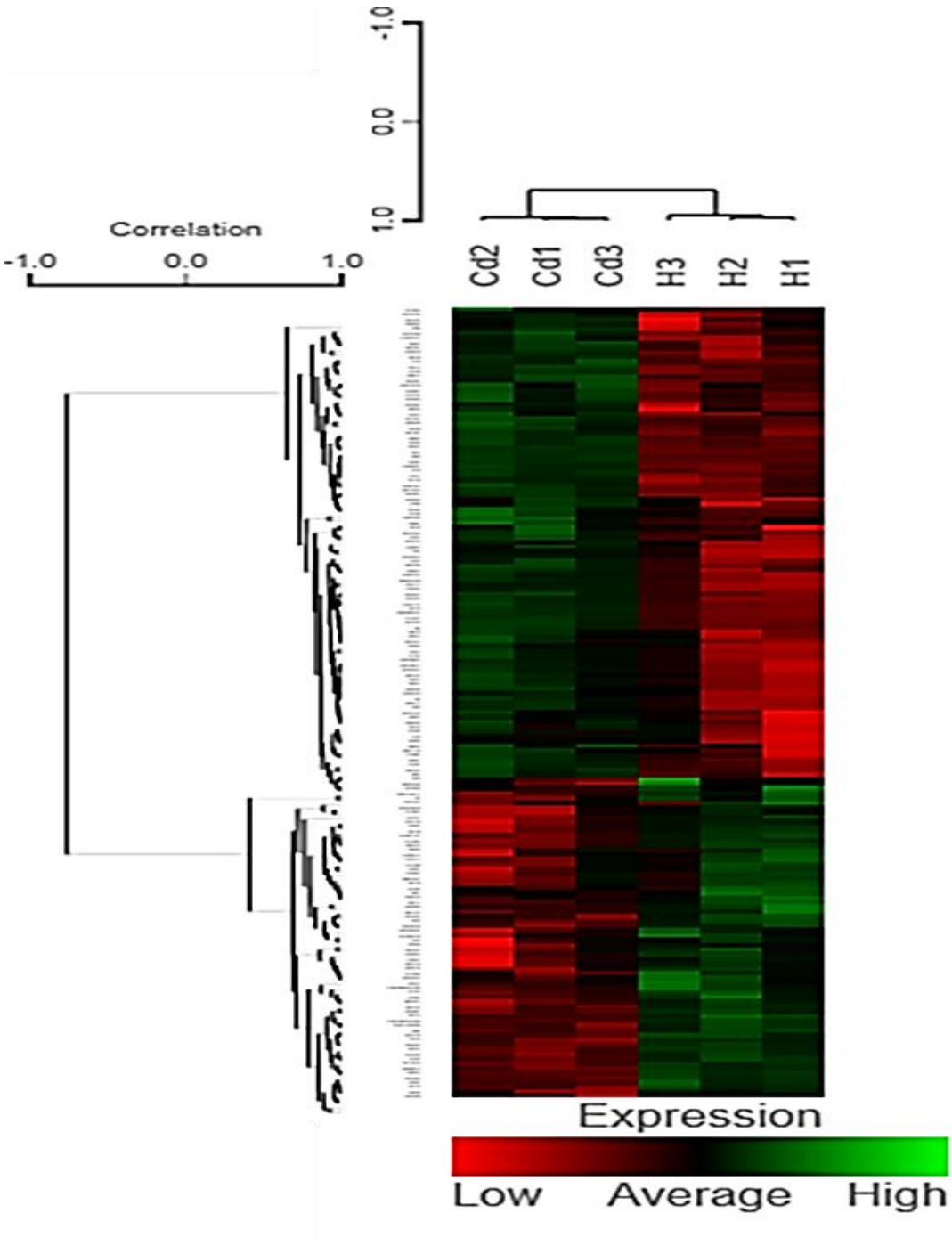

Fig. 6.12: Paired analysis of hierarchical clustering of all statistically significant differentially expressed genes between Cd only treated replicates and No Cd/No Se (shown as H in Fig. 6.12) treated replicates (n=3).

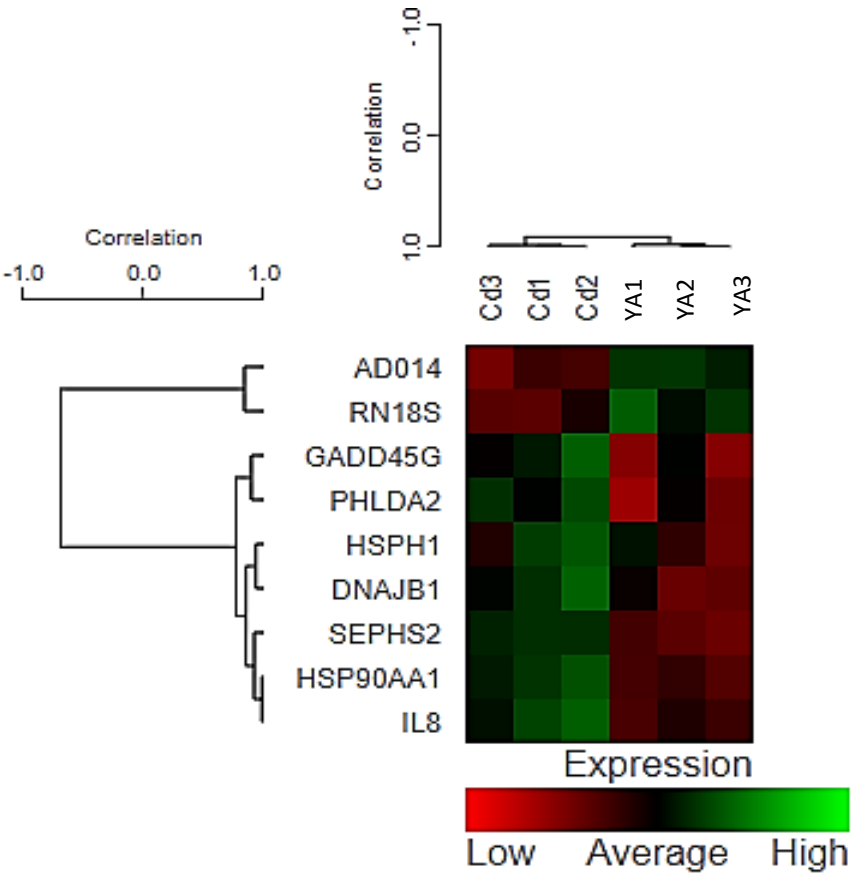

Fig. 6.13: Paired analysis of hierarchical clustering of all statistically significant differentially expressed genes between Cd only treated replicates and Cd + Se-YA treated replicates (n=3).

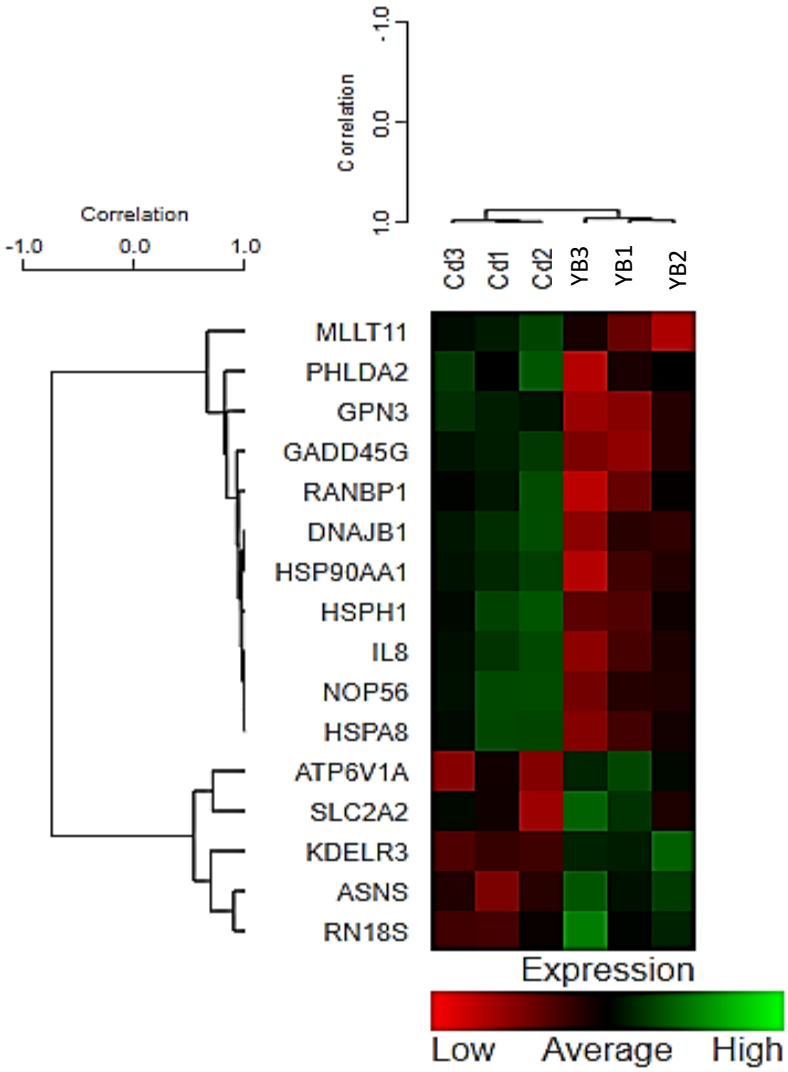

Fig. 6.14: Paired analysis of hierarchical clustering of all statistically significant differentially expressed genes between Cd only treated replicates and Cd + Se-YB treated replicates (n=3).

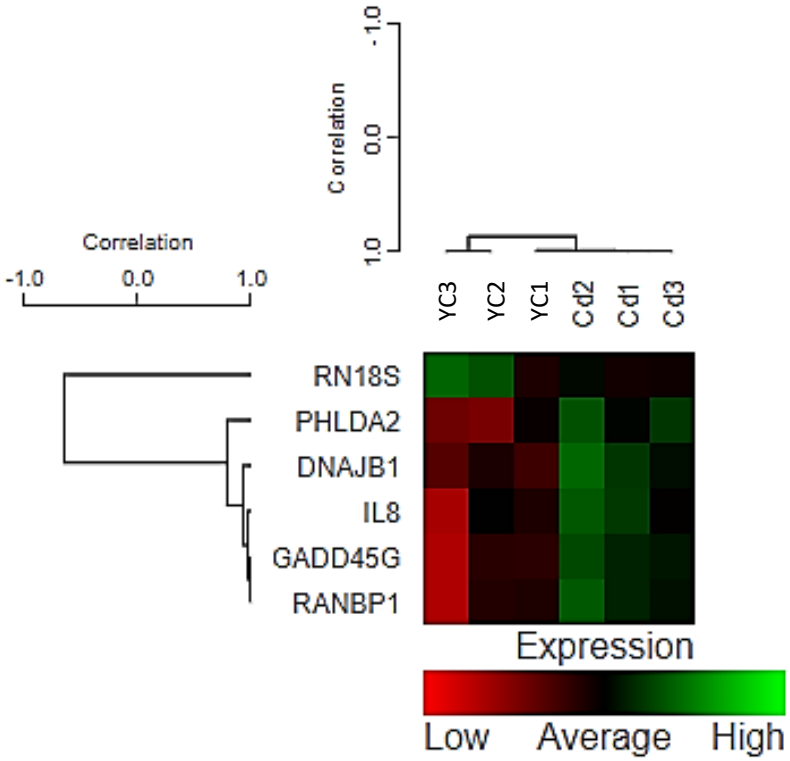

Fig. 6.15: Paired analysis of hierarchical clustering of all statistically significant differentially expressed genes between Cd only treated replicates and Cd + Se-YC treated replicates (n=3).

Table 6.1: Complete list of differentially expressed genes detected between Cd/No Se treatment and No Cd/No Se treatment (compiled according to q value significance).

Gene	log2(No Cd/No Se)	log2(Cd/No Se)	log2(Ratio)	q Value
ACLY	4.46	5.86	-1.4	1.39E-03
AIMP1	6.82	5.68	1.14	1.39E-03
ARL6IP5	6.97	5.88	1.09	1.39E-03
ASNS	7.93	6.78	1.14	1.39E-03
BNP	-10	6.07	-16.07	1.39E-03
C-JUN	8.01	7.1	0.91	1.39E-03
CCNG1	8.02	7.32	0.7	1.39E-03
CD70	8.03	6.87	1.16	1.39E-03
CLDN4	10.58	9.86	0.72	1.39E-03
CLU	3.08	5.3	-2.22	1.39E-03
DNAJA1	6.79	7.6	-0.81	1.39E-03
DNAJB1	5.71	7.55	-1.84	1.39E-03
FASN	5.93	7.14	-1.21	1.39E-03
FKBP4	6.62	7.84	-1.22	1.39E-03
FOS	7.97	6.32	1.65	1.39E-03
GLRX	8.04	9.12	-1.08	1.39E-03
HADHA	7.11	5.87	1.24	1.39E-03
HMGCS1	4.86	5.87	-1	1.39E-03
HNRNPAB	7.61	8.46	-0.85	1.39E-03
HO2	4.99	6.06	-1.07	1.39E-03
HSP90AA1	9.3	10.84	-1.54	1.39E-03
HSP90B1	8.91	9.85	-0.93	1.39E-03
HSPA8	8.08	9.83	-1.74	1.39E-03
HSPH1	4.72	6.99	-2.27	1.39E-03
IL8	3.19	6.7	-3.51	1.39E-03
INSIG1	3.19	4.85	-1.66	1.39E-03
KLF2	7.78	6.73	1.05	1.39E-03
MANF	6.96	7.85	-0.88	1.39E-03
MKI67	5.92	6.75	-0.83	1.39E-03
MT1A	5.93	11.41	-5.48	1.39E-03
MYBL2	5.7	6.56	-0.86	1.39E-03
NAMPT	4.75	5.89	-1.14	1.39E-03
NOP56	6.32	7.32	-0.99	1.39E-03
NPC1	2.65	4.07	-1.42	1.39E-03
NQO1	4.83	5.86	-1.03	1.39E-03
PCK2	5.57	4.28	1.3	1.39E-03
PCNA	6.9	7.75	-0.86	1.39E-03
PDIA4	6.78	7.6	-0.82	1.39E-03
PLIN2	5.92	4.57	1.35	1.39E-03

PTBP1	6.5	7.3	-0.8	1.39E-03
RANBP1	6.67	7.89	-1.22	1.39E-03
RENBP	5.3	3.58	1.72	1.39E-03
RN18S	9.52	8.57	0.96	1.39E-03
S100A14	8.6	7.66	0.95	1.39E-03
SCD	6.72	7.55	-0.83	1.39E-03
SEC23B	5.8	6.64	-0.84	1.39E-03
SLC2A2	7.25	6.43	0.82	1.39E-03
SMTN	4.26	5.26	-1.01	1.39E-03
SRSF2	6.29	7.52	-1.22	1.39E-03
TFRC	6.1	6.87	-0.78	1.39E-03
TUBB2A	8.1	8.82	-0.72	1.39E-03
TXNRD1	4.92	6.85	-1.92	1.39E-03
ACSL1	5.12	4.15	0.97	2.48E-03
AREG	5.89	6.78	-0.89	2.48E-03
BHLHE40	7.7	7.03	0.67	2.48E-03
MLLT11	3.58	5.46	-1.88	2.48E-03
SH3GLB1	6.84	5.84	0.99	2.48E-03
SSR1	5.13	5.89	-0.77	2.48E-03
C1H14orf166	8.07	7.35	0.72	3.33E-03
ECI2	4.06	5.33	-1.27	3.33E-03
FTH1	10.6	11.3	-0.7	3.33E-03
LOC100512700	7.01	6.12	0.89	3.33E-03
RGS2	5.32	6.36	-1.04	3.33E-03
RSF1	4.41	3.48	0.92	3.33E-03
SFN	8.21	8.95	-0.75	3.33E-03
ACTN1	8.99	9.65	-0.66	4.06E-03
AK3L1	5.04	5.84	-0.79	4.06E-03
EIF4G1	6.57	7.19	-0.62	4.06E-03
NR2F2	4.98	3.96	1.02	4.06E-03
OGT	4.74	4	0.73	4.06E-03
PN-1	7.13	7.8	-0.67	4.06E-03
CARKD	6.19	5.36	0.83	4.80E-03
EDN1	8.3	7.66	0.64	4.80E-03
HK2	1.44	4.07	-2.63	4.80E-03
OCLN	7.22	6.52	0.7	4.80E-03
CSE1L	6.94	7.53	-0.6	5.61E-03
DNTTIP2	4.82	5.64	-0.82	5.61E-03
PFKM	2.92	4.28	-1.36	6.47E-03
CCT3	7.15	7.77	-0.61	7.20E-03
TCP1	6.85	7.52	-0.67	7.20E-03
DKK3	7.96	7.3	0.65	7.72E-03
HES1	7.84	7.14	0.71	7.72E-03
MAL2	8.48	7.86	0.62	7.72E-03

OPTN	6.74	6.07	0.67	7.72E-03
E4	9.66	8.48	1.19	8.38E-03
IGFBP7	9.03	8.44	0.6	8.38E-03
MMP7	5.71	6.56	-0.85	9.11E-03
LOC100517160	8.27	7.65	0.62	1.21E-02
SOD2	4.13	5.18	-1.05	1.21E-02
HSPE1	8.08	8.76	-0.68	1.27E-02
SMAD7	4.21	3.09	1.11	1.27E-02
PSMG1	4.97	6.02	-1.05	1.32E-02
ZNF217	4.23	3.47	0.75	1.32E-02
EIF4A1	8.35	8.93	-0.58	1.38E-02
TIMP1	7.94	7.25	0.69	1.67E-02
ACSL3	6.68	6.04	0.63	1.73E-02
ARF5	6.17	4.92	1.25	1.73E-02
CLDN6	8.7	8.1	0.6	1.73E-02
EMG1	6.62	7.32	-0.7	1.73E-02
F3	10.08	10.65	-0.57	1.73E-02
FASTK	4.53	3.25	1.28	1.73E-02
GPI	7.6	8.15	-0.55	1.73E-02
HNF1B	4.81	3.96	0.85	1.73E-02
LRRC59	6.07	6.66	-0.59	1.73E-02
MCL1	7.32	6.74	0.59	1.73E-02
PLCD1	5.95	5.29	0.66	1.73E-02
SLN	4.69	6.15	-1.46	1.73E-02
SNRPA	4.63	5.88	-1.24	1.73E-02
EAF1	4.53	5.21	-0.69	1.75E-02
PEMT	5.53	9.01	-3.48	1.75E-02
SEC24A	4.22	4.9	-0.68	1.75E-02
MDH2	6.97	7.54	-0.57	1.87E-02
GSS	5.95	5.25	0.7	1.90E-02
SBDS	5.8	6.45	-0.65	1.90E-02
NOP2	3.98	4.81	-0.83	1.93E-02
VRK1	3.13	4.59	-1.45	1.93E-02
EIF4A2	8.03	7.49	0.54	2.03E-02
DNMT1	3.44	4.17	-0.73	2.10E-02
NSFL1C	6.08	6.74	-0.66	2.10E-02
PPID	3.88	5	-1.12	2.10E-02
LAT2	4.52	3.29	1.23	2.14E-02
RPL15	6.89	6.28	0.6	2.19E-02
CRAT	5.98	6.56	-0.59	2.23E-02
CYCS	6.23	6.88	-0.66	2.25E-02
FADS3	3.18	4.38	-1.21	2.25E-02
DHX15	5.11	5.74	-0.63	2.29E-02
STARD10	6.41	5.79	0.63	2.33E-02

TKT	6.86	7.41	-0.55	2.36E-02
BZW1	6.89	7.46	-0.57	2.40E-02
BTG2	4.77	3.97	0.8	2.44E-02
CCND3	6.17	5.13	1.05	2.57E-02
PPP1R10	4.21	4.94	-0.72	2.57E-02
SEPHS2	5.82	6.45	-0.63	2.76E-02
PRDX3	6.03	6.66	-0.63	2.80E-02
NDUFS5	7.51	6.66	0.85	2.88E-02
GRB10	6.92	4.49	2.42	3.34E-02
SRRM2	4.9	5.42	-0.52	3.47E-02
SMARCD2	4.32	3.42	0.9	3.50E-02
BRIX1	4.78	5.62	-0.84	3.63E-02
LPAR1	4.4	3.36	1.04	3.63E-02
SQLE	5.55	6.13	-0.58	3.63E-02
AURKA	4.54	5.45	-0.91	3.70E-02
EFNA1	6.69	5.76	0.93	3.73E-02
FAS	4.41	3.54	0.87	3.85E-02
CHCHD3	4.7	5.47	-0.77	3.98E-02
SNRPC	6.06	6.81	-0.75	4.24E-02
CDKN2B	6.92	5.25	1.67	4.28E-02
DNAJB11	5.83	6.42	-0.59	4.28E-02
PTMA	9.6	10.14	-0.54	4.35E-02
BMP3	5.33	4.61	0.72	4.46E-02
LOC414417	7.04	6.19	0.85	4.46E-02
TGFB1	6.18	5.62	0.56	4.46E-02
PCBP2	8.35	7.87	0.48	4.47E-02
ELAC2	4.47	5.39	-0.93	4.49E-02
AKAP2	4.28	4.83	-0.54	4.57E-02
CD9	8.78	8.29	0.49	4.57E-02
PPAN	3.58	4.58	-1	4.59E-02
PIK3AP1	4.26	3.63	0.63	4.61E-02
PSME3	5.59	6.4	-0.8	4.74E-02
SDCBP2	7.81	7.3	0.51	4.74E-02
SGK1	5.27	5.84	-0.57	4.74E-02
GGTA1	6.51	5.88	0.63	4.80E-02
AP3M1	4.36	4.97	-0.61	4.82E-02
NDUFS3	6.31	6.93	-0.62	4.82E-02
RPL30	10.76	10.28	0.48	4.82E-02
VDAC1	7.44	7.97	-0.53	4.82E-02
TGIF1	6.84	6.29	0.55	4.83E-02