L'effet anticancéreux d'un sélénium : étude de son rôle dans l'activité de réparation de l'ADN et la résistance au stress oxydant

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« Omnia venenum sunt: nec sine veneno quicquam existit.
Dosis sola facit, ut venenum non fit»[i]

[i] Paracelso from "Responsio ad quasdam accusationes & calumnias suorum aemulorum et obtrectatorum. Defensio III. Descriptionis & designationis nouorum Receptorum"

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Abbreviations

3′UTR : 3′ untranslated region

8-oxoGua : 7,8 dihydro-8-2′-desoxyguanine

6-4 PP : Pyrimidine (6-4) Pyrimidone Photoproduct

Ψ : Pseudouridine

AP : Apurinic/apyrimidinic

BER: Base Excision Repair

BSA: Bovine Serum Albumin

CPD: Cyclobutane Pyrimidine Dimer

DIO : Iodothyronine deiodinase

DMSe : Dimethylselenide

DMSO: Dimethylsulfoxyde

DR: Direct Reversal

DSB : Double Strand Breaks

DTT: Dithiothreitol

eFSec: Sec-specific elongation factor

ER: Endoplasmatic Reticulum

FapyG: 2,6-diamino-4-hydroxy-formamidopyrimidine

FEN1: Flap Endonuclease 1

Fpg: Formamidopyrimidine DNA glycosylase

GG-NER: Global Genome-NER

Grx: Glutaredoxin

GSH: reduced glutathione

GSSeSG: selenodiglutathione

GSSG: oxidized glutathione

GPx: Glutathione Peroxidase

Gy: Gray

H_2O_2 : Hydrogen Peroxyde

HCR: Host Cell Reactivation

HEPES: acide 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

HR: Homologous Recombinaison

JNK: c-Jun N-terminal Kinase

I^6A: N^6-isopentyladenosine

LOH: Loss of Heterozygosity

m^1A: 1-methyl adenosine

mcm^5U: 5-methylcarboxymethyluridine

mcm^5Um: 5-methylcarboxymethyluridine-'-O-methylribose

Met: Methionine

MMR: MisMatch Repair

MMS: methylmethane sulfonate

MSA^{IV}: Methylselenonic acid

MSA^{VI}: Mathylseleninic acid

MsrB1: methionine-R-sulfoxide B1

MTT: 3-(4,5-diméthylthiaeol-2-yl)-2.5-diphenyltetrazolium bromide

NADPH: β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced

N^3MeA: N3-methyladenine

N^3MeG: N3-methylguanine

N^7MeG: N7-methylguanine

NER: Nucleotide Excision Repair

NHEJ: Non Homologous End-Joining

NLS: Nuclear Localization Sequence

NMSC: NonMelanoma Skin Cancer

NPC: Nutritional Prevention of Cancer

MYH: A/G-specific adenine DNA glycosylase

O^6MeG: O6-methylguanine

OGG1: 8-Oxoguanine glycosylase

ODN: oligonucleotide

PARP : Poly(ADP-Ribose)Polymerase

PBS: Phosphate Buffer Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

RDA: Recommended Daily Allowance

ROS: Reactive Oxygen Species

SBP2: SECIS-binding protein 2

Se: Selenium

SECIS: SelenoCysteine Insertion Sequence

Sec: Selenocysteine

SELECT: Selenium and Vitamin E Cancer Prevention Trial

SeMSC: Se-methylselenocysteine

SM: Selenomethionine

SOD: Superoxide Disutase

SPS: selenophosphate synthetase

SSB: Single Strand Break

SS : Sodium Selenite

SV40: Simian Virus 40

TC-NER: Transcription Coupled-NER

$tRNA^{Sec}$: transport RNA of Sec

TMSe: Trimethylselenonium

Trx: Thioredoxin

TrxR: Thioredoxin reductase

UNG: Uracil-DNA glycosylase

UV: ultraviolet

XRCC1: X-Ray Cross-Complementation Protein 1

Chapter 1 - Review of literature

1. The two faces of selenium

"Vero è che li viandanti che passano de lì non ardiscono andare a que' monti con altre bestie che di quella contrada, perché vi nasce un'erba venenosa, di sorte che, se le bestie ne mangiano, perdono l'unghie, ma quelle di detta contrada cognoscono l'erba e la schifano di mangiare". (Il Milione, cap. XLIX).

In 1295, Marco Polo, during his travel along the Silk Road in the Suzhou region of western China, described that his horses lost hair and hooves because they had eaten poisonous grass. The toxic symptoms described by Marco Polo were supposed, later, to be typical of selenosis caused by having eaten selenium-accumulating vegetation.
Selenium (Se, from Greek, σελήνη, Selene which was the Goddness of the Moon on Greek mithology) was identified by Jöns Jacob Berzelius, a Swedish chemist, in 1817 during the oxidation of sulfur dioxide from copper pyrites in the production of sulfuric acid (Hatfield et al., 2001).
In the early years of this century, selenium was identified as the active principle in forages that caused livestock poisoning in South Dakota, which had first been recognized many years earlier by Madison, an Army doctor. This discovery led to investigations of natural sources of selenium, in the course of which Beath, at the University of Wyoming, noted the existence of a peculiar group of plants having the ability to accumulate quantities of selenium up to several thousand parts per million when grown on seleniferous soil (Beath, 1936). In 1937, Moxon published a report at the South Dakota Agricultural Experiment Station, in which he identified selenium as the toxic principle in some livestock-poisoning plants on the Western ranges (Moxon, 1938). The livestock problem, mistakenly called "alkali disease," occurred in acute form following the consumption by range animals of some wild vetches of the

genus *Astragalus*, which accumulated toxic amounts of selenium from the soil (O'Toole and Raisbeck, 1995).

In 1957, a German biochemist, Klaus Schwarz, working at the National Institutes of Health in Bethesda, published a paper that changed forever the public conception of selenium. He had thus identified the ability of selenium to protect against dietary liver necrosis, and this led shortly to selenium being recognized as an essential trace mineral nutrient (Schwarz and Foltz, 1958).

The U.S. Food and Drug Administration (USFDA) had to be convinced that selenium was safe at the recommended supplementary levels. USFDA was in particular concerned by works suggesting that selenium was carcinogenic. These studies involved rats that were fed diets inadequate in protein, to which selenium was added at levels much higher than needed to correct a selenium deficiency (Oldfield, 1987). The hypothesis was thus unsupported, and in 1973 scientists at the National Cancer Institute and the USFDA issued a joint statement declaring that "judicious administration of selenium derivatives to domestic animals would not constitute a carcinogenic risk".

Selenium was recognized as an important micronutrient for both humans and animals and is obtained through the diet from several sources including cereals, grains, nuts, vegetables, meat, and seafood (Pennington and Schoen, 1996). The recommended daily allowance (RDA) for selenium ranges from 55 µg/day to an upper limit of 350-450 µg/day intake comes from dietary supplementation and foods rich in this mineral (Burk, 2002; Schrauzer, 2001). The average intake of selenium for the development of symptoms due to deficiency is below 10 µg/day with symptoms such as congestive heart failure, stroke, or sudden death (Tapiero et al., 2003). Intake of selenium higher than the upper limit range 350-450 µg/day of RDA is also of major concern for humans and animals since it can result in selenium toxicity or selenosis. In humans, sign of selenosis include garlic breath, hair and nail loss, teeth deformation, skin lesions, and lowered haemoglobin levels upon dietary selenium intake of 5 mg/day (Yang et al., 1983). The delicate balance of the double selenium effect is synthesized in Figure 1.

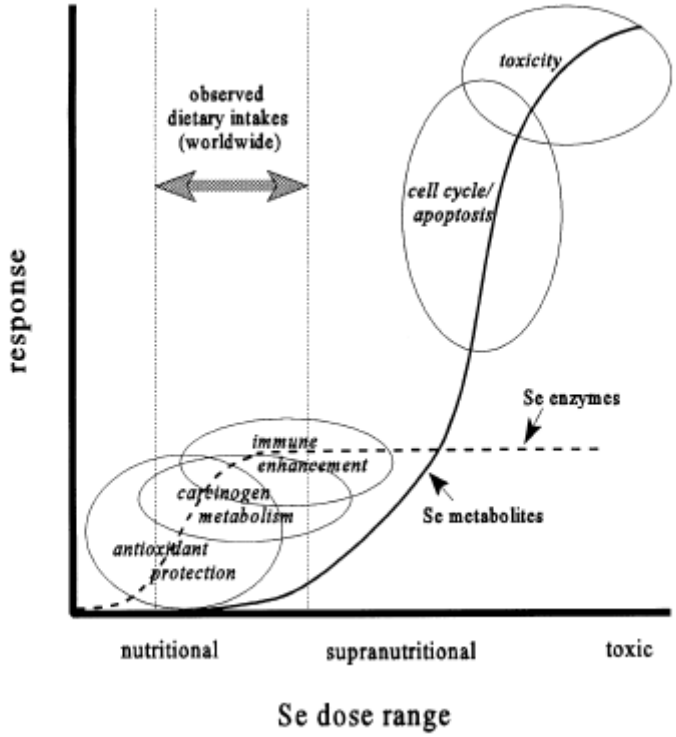

Figure 1: Two-stage model for the roles of Se in cancer prevention (Combs and Gray, 1998). Positive effects are mediated by selenoenzymes while toxicity results from same metabolites produced at high dose.

The selenium soil content may greatly vary depending on geographical location and mineral composition. In many regions of the world Se soil levels reflect the Se status of human populations (Diplock, 1993). There are some zones where Se levels in soil are very low (<0.05 ppm), such as parts of China, Finland and New Zealand. In these regions, diseases caused by Se deficiency in livestock and the effects on human health are well known. Nevertheless, in regions of high Se soil concentrations (>5 ppm), there is a net excess of this element as observed in Canada, Ireland, some regions of the western USA, and some zones of China and Germany (Navarro-Alarcon and Cabrera-Vique, 2008).

Therefore, it is important to know its abundance or deficiency in food and diet to determine the correct intake of Se in human beings and animals. Se bioavailability is affected by its chemical form (Thomson, 2004).

1.1 Physical-chemical properties of elemental selenium

Selenium is a metalloid found in group VIa of the periodic table, as are sulphur and tellurium. Se closely resembles sulphur in chemical properties with respect to atomic size, bond energies, ionisation potentials and electron affinities. The major difference between two these elements is that Se exists as reduced quadrivalent form whereas sulphur occurs as oxidised quadrivalent form. In addition, there is also a difference in acid strengths between these two elements (Tinggi, 2003).

The atomic weight of selenium is 78.96 g/mole. It has five naturally occurring stable isotopes and two beta particle emitting radioactive isotopes, ^{75}Se and ^{79}Se. It has four oxidation states in nature: -2, 0, +4, and +6, of which the +4 and +6 form the oxyanions selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), respectively in aqueous solution. In Table 1 is summarized the other atomic properties of selenium.

Atomic Properties and electronic configuration of selenium	
Atomic weight	78,96
Atomic number	34
Electronic configuration	$(Ar)3d^{10}4s^24p^4$
Common oxidative states	-2, 0, +4, +6
Ionization potential eV	9,75
Electron affinity eV	-4,21
Electronegativity	2,55
Polarizability	105

Table 1: Physico-chemical properties of selenium

1.2 Metabolism of selenium compounds

The level of Se in plants and in turn in animals depends on the amount of biologically available Se in the soil (Navarro-Alarcon and Cabrera-Vique, 2008). Both inorganic and organic forms of selenium can be utilized as nutritional and supplemental selenium sources; selenate and selenite (Figure 2) are typical inorganic ones (Whanger, 2002).

Figure 2: Chemical structures of sodium selenite (SS) and sodium selenate

In addition, organic selenium is present in general as selenoamino acids, which can be divided into several groups (Figure 3). Selenocysteine (Sec) and selenomethionine (SM) are present as amino acids constituents in gene products, selenoproteins and general proteins respectively. The principle chemical form of selenium in animal tissues is selenocysteine, unlike plant in which selenomethionine predominates. Se-Methylated SM and Se-methylselenocysteine (SeMSC) are chemically less reactive forms compared to the corresponding non-Se-methylated selenoamino acids, and are accumulated in selenium-accumulators in the forms of SM bound to macromolecules such as in grains, mushrooms and yeast, and in the form of free SeMSC in garlic and onion (Suzuki et al., 2007).

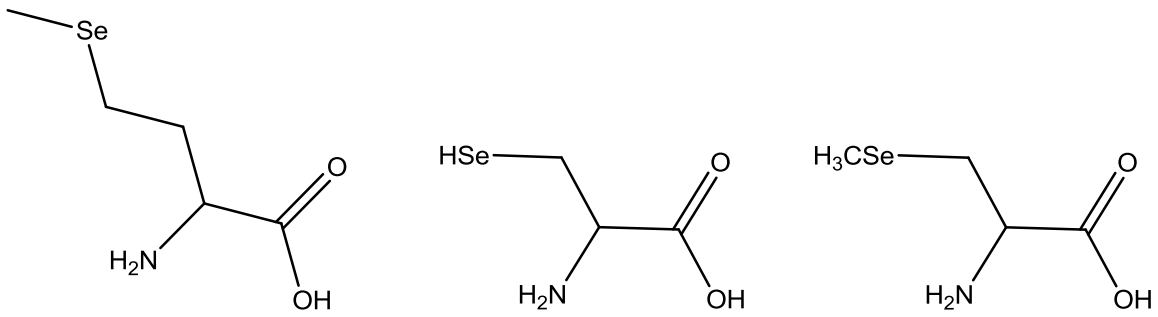

Figure 3: Chemical structures of SM, Sec and SeMSC respectively

In addition to these typical natural nutritional selenium sources, non-natural methylselenonic ($CH_3Se(O)_2OH$; MSA^{IV}) and methylseleninic acids ($CH_3Se(O)OH$; MSA^{VI}) have been shown to be incorporated into selenoproteins and excretion metabolites selenosugars and trimethylselenonium (TMSe), suggesting that MSA^{IV}

and MSA^{VI} could be regarded as a third group of nutritional and supplemental selenium sources (Figure 4) (Suzuki et al., 2006a).

Figure 4: Chemical structures of MSA^{VI} and MSA^{IV} respectively

Once it has been ingested, selenium is distributed within the body. Its absorption and excretion depend on several factors, particularly the chemical form as well as the total amount of the element in the diet. In addition, intake can be affected by the presence of other components of food, including sulfur, heavy metals, and vitamins (Navarro-Alarcon and Cabrera-Vique, 2008).

Absorption of selenium occurs mainly at the lower end of the small intestine. In general, organic compounds, such as SM, are absorbed more efficiently than are inorganic forms, particularly selenite, with uptake from the gastrointestinal tract of more than 90% of SM compared to about 60% of selenite (Reilly, 2006). Differences in chemical form also affect levels of retention in the body over time. It has been shown, in humans as well as in experimental animals, that selenomethionine is retained more efficiently than selenite or selenate, but is not as efficient in maintaining selenium status (Fairweather-Tait, 1997). It has also been reported that selenium is more readily available if it is in plants rather than in animal products (Young et al., 1982).

All inorganic and organic nutritional selenium sources have been shown to be used for the synthesis of selenoproteins or excreted as selenosugars. It seems thus likely that a common metabolite selenide (HSe^-) is generated (Suzuki and Ogra, 2002), as schematically shown in Figure 5.

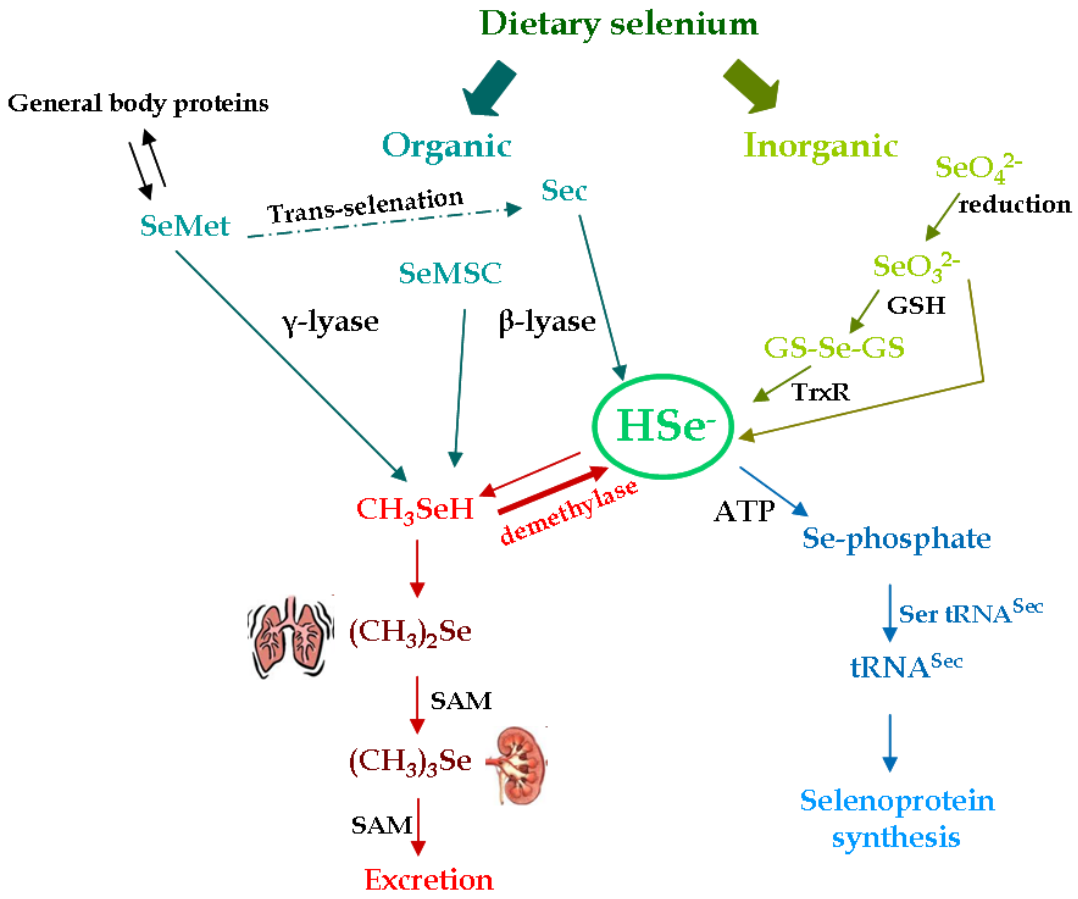

Figure 5: Simplified mammalian selenium metabolism. Selenium is normally taken up from the diet mainly as SM, Sec, selenate and selenite. Namely, inorganic selenium is reduced to selenide. Sec liberated from selenoproteins is transformed to selenide by β-lyase. SM liberated from general proteins and of free SM sources is transformed into selenide either through methylselenol by γ-lyase followed by demethylation (SM→CH_3SeH→H_2Se), or through Sec by β-lyase after the trans-selenation pathway (SM→Sec→H_2Se). SeMSC is transformed into methylselenol by β-lyase. Methylselenol is demethylated to selenide for further utilization for selenoprotein synthesis and/ or for excretion in the form of selenosugar. At the same time, methylselenol is further methylated to dimethylselenide (DMSe) and trimethylselenonium (TMSe) for excretion.

Selenate is reduced into selenite by glutaredoxin (Grx) and thioredoxin (Trx) in the presence of GSH (Bjornstedt et al., 1997). Glutathione (GSH) is considered to be the main component of the Se metabolism pathway taking part in the first of a series of reduction reactions which convert selenite to hydrogen selenide (H_2Se). Selenite forms a covalent adduct with glutathione (GSH) in the form of selenodiglutathione (GS-Se-SG), which has been proposed to be the major metabolite of inorganic selenium compounds in mammalian tissues. This reduction pathway is tightly connected to production of the superoxide radical ($O_2^{\bullet -}$) (Wachowicz et al., 2001).

Sec is transformed into selenide through the β-lyase reaction, whereas SM is transformed into selenide through the trans-selenation pathway into Sec followed by the β-lyase reaction. However, it was proposed that excessive SM is directly transformed through the γ-lyase reaction into methylselenol followed by the demethylation reaction. Intact SM is incorporated exceptionally into proteins without being discriminated from methionine (Met) (Suzuki, 2005).

A monomeric form of SeMSC is assumed to be transformed into selenide through the β-lyase reaction to produce methylselenol, and then into selenide through demethylation (Suzuki et al., 2007).

In addition to these inorganic selenium and organic selenoamino acids, a third group of selenocompounds i.e., MSA^{VI} and MSA^{IV}, has been proposed to be reduced to methylselenol, and then transformed through demethylation into selenide. Methylselenol is, thus, thought to be the key intermediate leading to selenide in the case of Se-methylated selenocompounds. At the same time, methylselenol is known to be an intermediate for further methylated metabolites, i.e., dimethylselenide (DMSe) and trimethylselenonium (TMSe), for the excretion of excessive selenium (Ohta and Suzuki, 2008). Under these circumstances, selenium is methylated and incorporated into selenosugars that can be excreted through urine. Excess levels of methylated selenium are emitted through the lungs (Reilly, 2006).

1.3 Selenoprotein synthesis

Selenium is an essential trace element for mammals and it is cotranslationally inserted into protein as the amino acid selenocysteine (Sec), the 21^{st} aminoacid. This aminoacid is an example of codon redefinition due to the fact that it is encoded by a UGA codon, which commonly specifies termination (codon stop). It is incorporated directly into polypeptide chain during translation in a number of bacteria, archaea and eukaryotes. Proteins that incorporate selenocysteine are called selenoproteins.

1.3.1 Structure-function of the tRNA^{Sec}

The Sec tRNA is the only known tRNA that governs the expression of an entire class of proteins, the selenoproteins. The Sec tRNA^{Sec} gene occurs in single copy in the genome of all mammals, it is 87 nucleotides long and a CCA terminus is added post-transcriptionally to make its final length of 90 nucleotides (the longest eukaryotic tRNA sequence). Transcription of Sec tRNA^{Sec} is also unusual as it begins at the first nucleotide within the coding sequence while all other tRNAs have a leader sequence that must be processed. Sec tRNA^{Sec}, therefore, has a 5' triphosphate in its terminal guanosine moiety (Hatfield and Gladyshev, 2002).

The tRNA^{Sec} has an 9-bp A-stem and 4-bp T-stem (a 9/4 secondary structure) forming a 13-bp long acceptor-TΨC helix; in contrast, canonical tRNAs forms a 12-bp acceptor-TΨC helix (7+5) (Hubert et al., 1998). Unlike other tRNAs, tRNA^{Sec} has a 6 bp extended D-stem, that was shown to be a major identity determinant for serine phosphorylation (Wu and Gross, 1994). It also only contains four modified bases, thus fewer than canonical tRNAs. The shared modified bases are N^6-isopentyladenosine (i^{6}A) at position 37, pseudouridine (ψ) at position 55 and 1-methyl adenosine (m^{1}A) at position 58 (Allmang and Krol, 2006) (Figure 6).

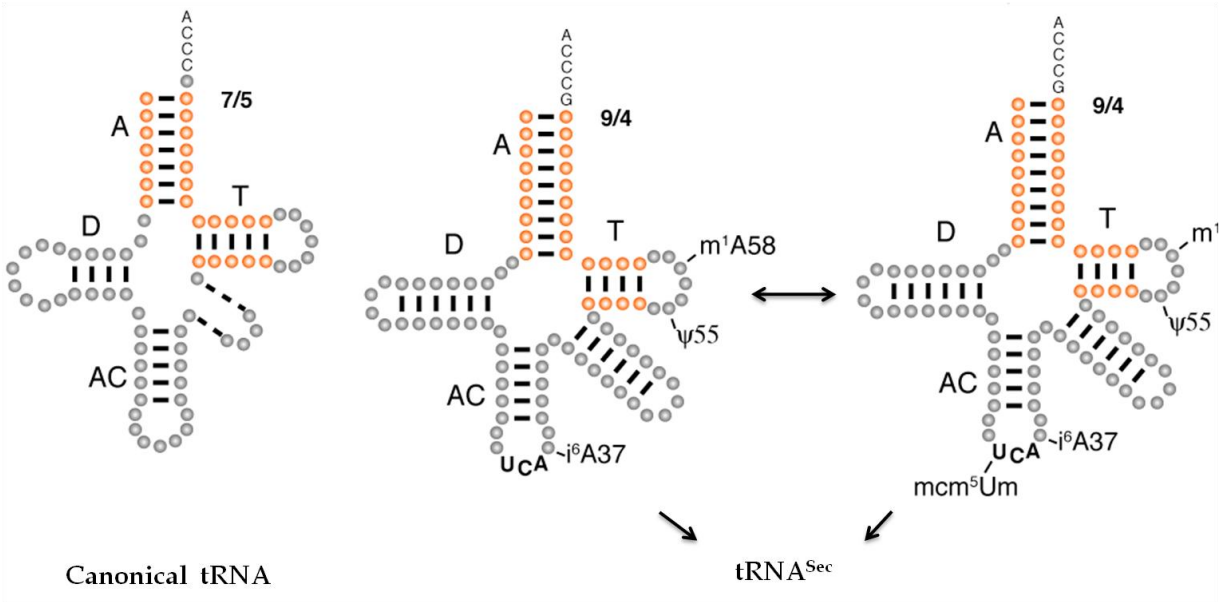

Figure 6: Cloverleaf models of mammalian canonical tRNA and two isoforms of mammalian tRNA^{Sec} mcm^{5}U and tRNA^{Sec} mcm^{5}Um.

The Sec $tRNA^{Sec}$ population consists in two major isoforms that differ from each other by a single methyl group in the wobble position of the anticodon. One form contains 5-methylcarboxymethyluridine (mcm^5U) and the other 5-methylcarboxymethyluridine-2'-O-methylribose (mcm^5Um) (Figure 6).

The post-synthesis of ψ and m^1A occur initially in the nucleus at position 55 and 58, respectively. The formation of mcm^5U and mcm^5Um at position 34 and i^6A at position 37 occurs in cytoplasm. The mcm^5U isoform is converted to the mcm^5U-i^6A form that in turn is converted to the mcm^5Um form (Hatfield and Gladyshev, 2002). Interestingly, the addition of this methyl group is responsive to selenium status (Hatfield et al., 1991) and its presence confers dramatic changes in tertiary structure (Diamond et al., 1993; Kim et al., 2000).

1.3.2 Biosynthesis of Sec

Selenocysteine does not occur as a free amino acid. The first step of its biosynthesis consists in the charge of serine on the specific $tRNA^{Sec}$ by the conventional seryl-tRNA synthetase. Interestingly, the serylated form can serve to suppress UGA termination codons *in vivo* and seryl- $tRNA^{Sec}$ is therefore an authentic nonsense suppressor tRNA. The seryl to selenocysteyl conversion occurs via the phosphoseryl intermediate that is phosphorylated, in the presence of ATP, by phosphoseryl-$tRNA^{Sec}$ (PSKT) (Carlson et al., 2004).

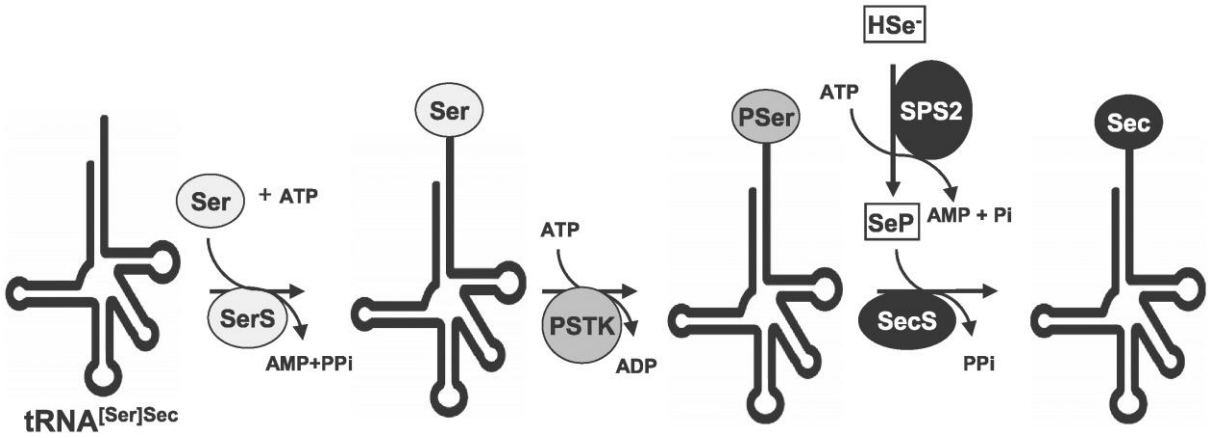

Figure 7: Sec biosynthesis in eukaryotes (Xu et al., 2007). $tRNA^{Sec}$ is first ligated with serine to form Ser-$tRNA^{Sec}$. The seryl moiety of Ser-$tRNA^{Sec}$ is then phosphorylated by PSTK to yield P-Ser $tRNA^{Sec}$, which is converted to $tRNA^{Sec}$ and used on the ribosome to insert Sec into a specific site in a nascent polypeptide of selenoproteins.

The active form of selenium that is donated to the intermediate in Sec biosynthesis had been identified as monoselenophosphate (Allmang and Krol, 2006), which is transferred to the phosphoseryl-tRNA^{Sec} to generate selenocysteyl-tRNA^{Sec}. Two selenophosphate synthetase genes in mammals, designed SPS1 and SPS2, have been identified and implicated as essential components in selenoprotein synthesis. SPS2 is a selenoprotein and thereby may autoregulate its own production along with the biosynthesis of other selenoproteins (Guimaraes et al., 1996). SPS2 also possesses a higher catalytic activity than SPS1. That has been speculated to mostly play a role in Sec recycling and selenium salvage (Tamura et al., 2004).

1.4 Mechanism of Sec insertion in eukaryotes

The fact that UGA has a dual role of serving as a stop and a Sec codon raises an important question how the cell distinguishes between these two functions. Besides Sec tRNA^{Sec} and the in-frame UGA codon in selenoprotein mRNA, there are several other factors that are required for the donation of Sec to protein and dictate the specific function of UGA as Sec. These include the (i) cis-acting stem-loop structure, designed the **S**elenoCysteine **I**nsertion **S**equence element; (ii) the SECIS-binding protein 2 (SBP2); (iii) and the Sec-specific elongation factor (eFSec) (Papp et al., 2007).

1.4.1 Cis-acting element: SECIS element

Eukaryotic SECIS element is located in the 3'untranslated region (3'UTR) of the mRNA. It is composed of two helixes separated by an internal loop; a SECIS core structure, Quartet, located at the base of helix 2; and an apical loop (Figure 8). The Quartet contains conserved GA/AG non Watson-Crick base pairs and is the main functional site of the stem-loop structure (Walczak et al., 1998). The presence of such a tandem GA base pairs constitutes a recurrent motif called the kink-turn (or k-turn). Two slightly different SECIS RNA secondary structure models could be derived. They only vary at the apex and give rise to type 1 or type 2. Type 2 SECIS possesses an additional helix 3 and a shorter apical loop, compared to type 1 (Grundner-Culemann et al., 1999).

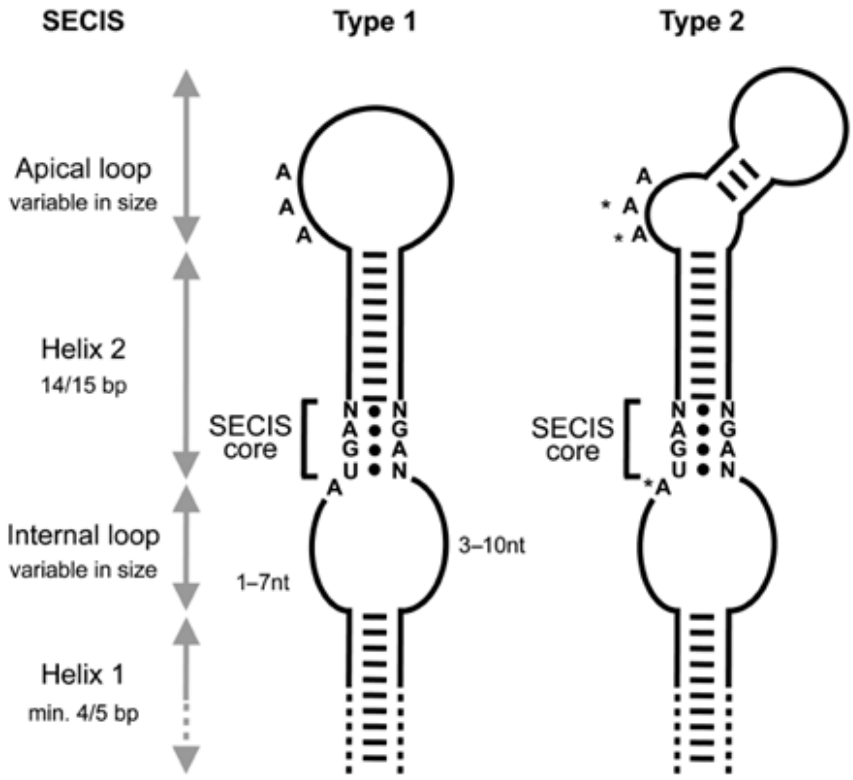

Figure 8: Schematic of eukaryotic SECIS elements (Latreche et al., 2009). The four main structural motifs are identified by arrows. A bracket indicates the position of the SECIS core that contains a quartet of non-Watson–Crick base pairs. Highly conserved nucleotides are represented in letters. The dots symbolize the two sheared tandem GA base pairs that are essential for kink-turn structures. The asterisks indicate the variations from the consensus sequence (AA) that are present as CC in several proteins SECIS elements.

These criteria have allowed the development of algorithms that can predict SECIS elements, and thus selenoproteins, from nucleotide databases (Kryukov et al., 1999).

It is demonstrated that as a SECIS element is moved from 111 to 60 to 51 nucleotides from a UGA codon, the Sec incorporation directed by that codon is drastically reduced (Martin et al., 1996). A likely explanation for this phenomenon is that steric constraints between the advancing ribosome and SECIS element recognition factors somehow preclude the formation of a productive interaction among factors required for Sec insertion at the UGA codon (Low and Berry, 1996).

Moreover, it is observed that selenoprotein open reading frames tend not to terminate in UGA codon. Such a configuration could present a problem, as it has been shown that any given codon, when mutated to UGA could act as Sec incorporation signal. One notable exception to this rule is selenoprotein W, which

terminates in UGA, but also has the unusual feature of a SECIS element in extremely close proximity, 51 nucleotides, to the termination codon (Whanger, 2000). It is precisely this feature that precludes Sec incorporation at the second UGA codon, resulting in termination of protein synthesis.

A single SECIS element is sufficient to dictate Sec incorporation in all selenoproteins with the exception of selenoprotein P, which contains two SECIS elements that direct the decoding of 10 UGA-Sec codons in mammal (Fixsen and Howard, 2010; Stoytcheva et al., 2006).

1.4.2 Trans-acting components

A bunch of proteic factors has been described to participate in selenoproteins synthesis. Several trans-acting factors are involved in the process, including the specific $tRNA^{Sec}$; the eukaryotic Sec-specific elongation factor (eEFsec); and SECIS binding protein 2 (SBP2). The list of protein factors involved in this mechanism is constantly growing, the most recent members being the ribosomal protein L30, soluble liver antigen (SLA), and tRNA selenocysteine 1-associated protein 1 (SecP43) (Figure 9).

SBP2 is one of the oldest and best-characterized factors in this process. It was shown to bind directly to SECIS elements via its L30-type RNA binding domain (Chavatte et al., 2005). SBP2 is proposed to function by recruiting eEFSec-$tRNA^{Sec}$ complex to the ribosome (Tujebajeva et al., 2000) and seems to play a role in dictating Sec incorporation efficiency (Low et al., 2000; Zavacki et al., 2003). One aspect of SBP2 regulation that has not been thoroughly addressed is its subcellular localization in cells. A putative nuclear localization signal (NLS) within the SBP2 primary amino acid sequence was reported early on (Copeland et al., 2000), leading to the hypothesis that SBP2 may translocate to the nuclear compartment. However, in later studies, endogenous SBP2 was detected just in the ribosomal fraction of cells and over-expressed SBP2 was detected within the cytoplasm of cells (Copeland et al., 2001; Kinzy et al., 2005), generating confusion about the localization of SBP2 in cells. Interestingly, SBP2 is stably associated with 80S ribosomes, and may be interacting through binding to 28S rRNA, independently of its interaction with the SECIS

element, suggesting that SBP2 may directly modify the coding potential of the ribosome to which it is bound.

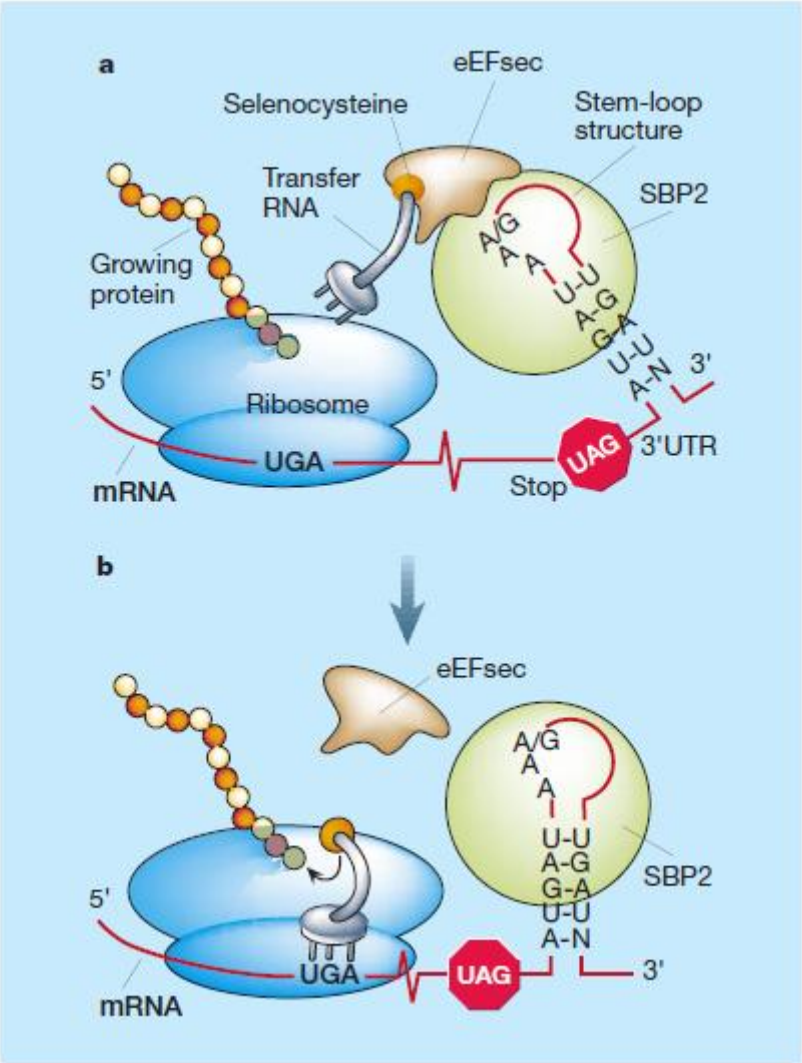

Figure 9: Redefining the stop codon in mammalian messenger RNAs (Atkins and Gesteland, 2000). The nucleotide sequence UGA normally specifies that the ribosome should stop translation. But sometimes this stop codon can be redefined, so that selenocysteine is incorporated instead. This model shows how this might be done. **a,** A 'stem loop' structure in the downstream, untranslated part of the mRNA binds to a protein called SBP2. SBP2 in turn binds to the eEFsec protein, which itself has recruited the transfer RNA carrying selenocysteine. **b,** The selenocysteine-bound tRNA is then delivered to the waiting UGA, for incorporation into the growing aminoacid string that constitutes the newly created protein.

The role of EFSec in Sec incorporation seems to be complex, as it is implicated in both the Sec-biosynthesis and Sec-incorporation pathways and appears to interact with most of the protein and RNA components of the selenoprotein-synthesis machinery. In terms of its role in selenoprotein translation, a model has been proposed in which ribosomal protein L30 binding to the SECIS element alters its structure, which would

induce SBP2 release, promoting the delivery of $tRNA^{Sec}$ to the ribosomal site (Chavatte et al., 2005). SecP43 and SLA play a role in the formation or stabilization of the EFSec–SBP2–Sec $tRNA^{Sec}$ complex and promote the formation and subcellular localization of the SPS1/SLA/SecP43 complex. This supramolecular protein complexe consisting of EFSec, SecP43, SLA, SPS1, SBP2 and L30 appears to form and dissociate dynamically in a nuclear, cytoplasmatic or ribosomal-specific manner (Atkins and Gesteland, 2000).

1.5 Selenoproteins

Nutrients in general, and selenium specifically, can impact gene expression potentially at six steps: transcription, nuclear processing, nuclear export, translation, mRNA stability and protein turnover. Selenium itself is a key regulator of its incorporation into selenoproteins and acts predominantly at transcriptional and post-transcriptional levels and through redox regulation (Driscoll and Copeland, 2003).

Animal studies as well as cell-culture models have demonstrated that a hierarchy in selenoprotein expression exists during selenium deprivation and repletion (Behne and Kyriakopoulos, 2001).

The selenium-containing proteins known so far can be divided into three groups: proteins into which the element is incorporated non-specifically, specific selenium-binding proteins, and specific proteins that contain selenium in the form of genetically encoded selenocysteine and that have been defined as selenoproteins (Lobinski et al., 2000).

At present, there are 25 human selenoprotein-encoding genes that have been identified, roughly half of which are only poorly characterized (Figure 10).

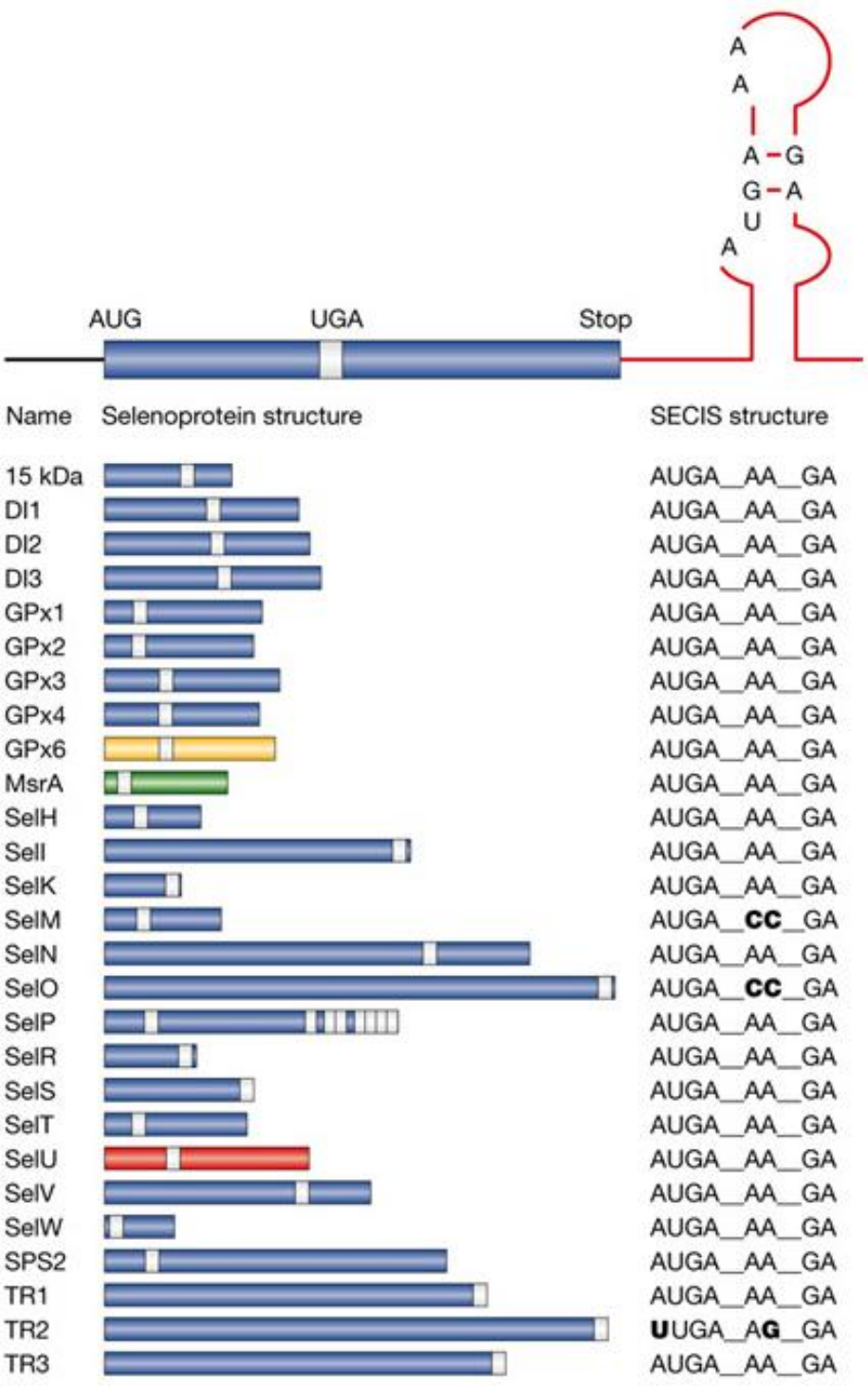

Figure 10: Animal Sec-containing proteins. All currently known selenoproteins are listed (left); locations of Sec in the selenoprotein sequences are indicated (white box) and SECIS structure (right).

1.5.1. Glutathione peroxidase

Glutathione peroxidase (GPx) was the first mammalian protein shown to incorporate selenium in the form of Sec into its catalytic site and was assumed to be associated

with the antioxidant activity of selenium (Forstrom et al., 1978). GPxs are well known for catalyzing the reduction of hydrogen peroxide and lipid hydroperoxides (LOOH), thus protecting cells from oxidative damage. In humans, seven GPxs are known, five of which are selenoenzymes and two that contain cysteine instead of Sec. The mechanism by which GPxs reduce their substrates is schematically presented in Figure 11. GPxs enzymes reduce hydroperoxides using glutathione (GSH) as a reducing agent. Hydroperoxides oxidize the Sec (selenolate) at the active site of GPx to selenoic acid. Two molecules of GSH are required to convert the oxidized GPx back to its active reduced state. GSH is a tripeptide (γ-glutamate, cysteine and glycine) that binds the reduced GPx adjacent to the catalytic centre. The thiol (SH) group from one GSH molecule binds with the oxidized selenium (selenoic acid) to form a selenosulfide bridge. A second GSH molecule is required to bind and split the bridge, regenerating selenolate and an active GPx enzyme. Glutathione reductase (GR) is then required to convert the oxidized glutathione (GSSG) back to its reduced form (GSH). GR utilizes electrons from NADPH, produced during glucose-6-phosphate oxidation (Reeves and Hoffmann, 2009).

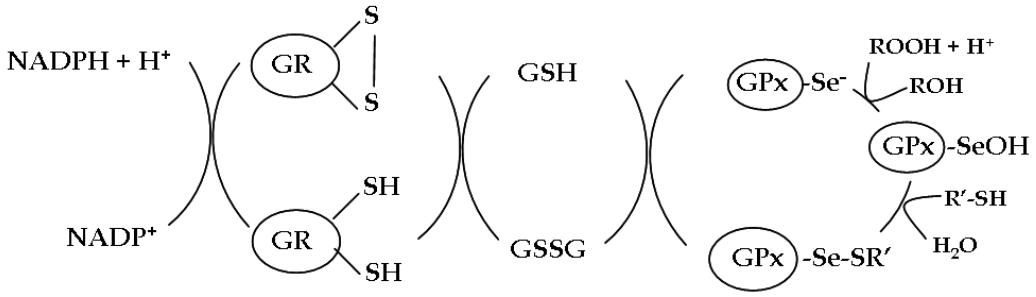

Figure 11: Schematic diagram of glutathione peroxidase system. Electron transfer from NADPH *via* glutathione reductase and GSH to mammalian glutathione peroxidases. GPxs obtain electrons from NADPH, *via* glutathione reductase (GR) and GSH to catalyze the reduction of hydrogen peroxide and organic hydroperoxides, thus protecting cells from oxidative damage.

The GPx family members include the following: the ubiquitously expressed cytosolic GPx (GPx1) (Flohe et al., 1973), a gastrointestinal-specific enzyme (GPx2) (Wingler and Brigelius-Flohe, 1999), a secreted protein found in plasma (GPx3) (Takahashi et al., 1990), a ubiquitously expressed enzyme that acts on oxidized lipids, phospholipid hydroperoxides glutathione peroxidase (GPx4), including a sperm nuclei–specific

enzyme (snGPx4) (Borchert et al., 2003), and a newly discovered glutathione peroxidase (GPx6) located in olfactory epithelium and embryonic tissues (Kryukov et al., 2003). The individual GPxs differ mainly by their preferred peroxide substrate, their tissue-specific expression pattern and the stimulus-dependent regulation of their biosynthesis (Brigelius-Flohe, 1999).

GPx1 is the most abundant and ubiquitously expressed selenoproteins. GPx1 is also one of the most highly sensitive to changes in Se status, with levels of mRNA and protein dramatically reduced under low Se conditions (Sunde et al., 2009). In addition to Se status, other factors like oxidative stress influence the expression of GPx1. Somewhat counter-intuitively, oxidative stress has been shown to reduce levels of GPx1 (Cheng et al., 1999).

This cytosolic enzyme can only metabolize hydrogen peroxide and some organic hydroperoxides, but not fatty acid hydroperoxide in phospholipids (Lei et al., 2007).

A role of GPx1 in protecting against certain cancers has been supported by several lines of evidence. GPx1 expression has been found to be decreased or repressed in various *in vitro* and *in vivo* models of cancer (Gladyshev et al., 1998a) and hypermethylation of the GPx1 promoter in gastric cancer cell lines has recently been demonstrated (Jee et al., 2009).

Transgenic mouse models involving deletion or overexpression of GPx1 have provided important insights into functional significance of proper levels of GPx1 expression. In general, GPx1 knockout mice are healthy and fertile with no apparent increased sensitivity to hyperoxia (Ho et al., 1997). When challenged with oxidative stress-inducing agents such as hydrogen peroxide, GPx1 knockout mice were found to be more susceptible to morbidity and mortality (Cheng et al., 1998). Also, GPx1 knockout mice were similar to Se deficient mice in their susceptibility to coxsackievirus-induced cardiomyopathy similar to human Keshan disease (Beck et al., 1998).

There have been several studies demonstrating associations between *GPx1* genetic polymorphisms and cancer, though not all have consistently supported significant associations (Arsova-Sarafinovska et al., 2009; Lei et al., 2007).

GPx2 is mainly expressed in the whole gastrointestinal tract including the squamous epithelium of the esophagus of healthy organisms and, in humans, is also detectable in liver (Wingler and Brigelius-Flohe, 1999). The role of GPx2 is mainly to protect intestinal epithelium from oxidative stress. GPx2 exhibits substrate specificity similar to that of GPx1 that includes hydrogen peroxide, tert-butyl hydroperoxide, cumene hydroperoxide, and linoleic acid hydroperoxide, but not phosphatidylcholine hydroperoxide (Chu et al., 1993). The location of GPx2 expression suggests that this selenoprotein may serve as a first line of defense against exposure to oxidative stress induced by ingested prooxidants or gut microbiota.

GPx3 is a secreted glycosylated protein and is the only secreted GPx enzyme. It constitutes approximately 20% of Se found in the plasma, though this number may change depending on Se status of the individual. An important role was identified for GPx3 in early experiments showing a role in regulating the bioavailability of nitric oxide (NO) produced from platelets and vascular cells (Freedman et al., 1995).

Unlike other GPxs, GPx4 can reduce phospholipid- and cholesterol-hydroperoxides directly, by using electrons from thiols protein as well as from glutathione in mammalian cells (Imai and Nakagawa, 2003). GPx4 is present in cytosolic, mitochondrial, and nuclear isoforms with differential tissue distribution. An additional fundamental role of GPx4 is its involvement in sperm maturation and male fertility. GPx4 exists as a soluble redox-active enzyme in spermatids and undergoes oxidative polymerization, forming a structural base of the sperm mitochondrial capsule in mature spermatozoa (Ursini et al., 1999). Severe and prolonged Se deficiency results in sterility as spermatogenesis was arrested, whereas in lower Se deprivation reduced sperm motility leading to impaired fertilization capacity and induced abnormal sperm morphology (Hatfield et al., 2001). A recent study showed that Se supplementation was highly effective in modulation of redox state in testicular cells (Erkekoglu et al., 2010) .

1.5.2. Thioredoxin reductase

The fact that mammalian TrxR is a selenoprotein was first discovered by Stadtman and co-workers in 1996 (Tamura and Stadtman, 1996). The selenocysteine-containing enzyme thioredoxin reductase (TR) catalyzes the NADPH-dependent reduction of thioredoxin (Gladyshev et al., 1996). The thioredoxin/ thioredoxin reductase system is widely used in biological systems to reduce ribonucleotides to deoxyribonucleotides, which are essential in the synthesis of DNA, to maintain redox balance in cells, to regulate activity of transcription factors, and to regenerate antioxidant systems.

Both Trx and TrxR are in mammals expressed as dedicated isoforms for either predominantly cytosolic (Trx1 or TrxR1) or mitochondrial (Trx2 and TrxR2) localization (Arner and Holmgren, 2000). In mammals a third form of TrxR is also expressed, predominantly in testis, which in contrast to TrxR1 and TrxR2 can reduce glutathione disulfide in addition to Trx. This enzyme has therefore been named TGR, indicating its thioredoxin/glutathione reductase activity (Sun et al., 2001). All three reductases (TrxR1, TrxR2 and TGR) are selenoproteins with their disulfide reductase activities involving a carboxylterminally located selenocysteine-containing active site (–Gly–Cys–Sec–Gly), present in these enzymes in a 16 residue elongation to a glutathione reductase-like scaffold structure (Lee et al., 2000).

The mechanism of TrxR-dependent reduction of substrates involves electron transfer from NADPH to FAD, *via* the N-terminal active site to the Cys-Sec selenenylsulfide bond within the C-terminal active site of the opposite subunit (Biterova et al., 2005). This is depicted in Figure 12. Because of the low pKa of the selenol group, it becomes ionized at physiologic pH to the reactive selenolate form, giving rise to a cysteinyl-selenol. Therefore, TrxR is a highly reactive enzyme that can also be easily attacked by electrophilic agents (Rundlof et al., 2000).

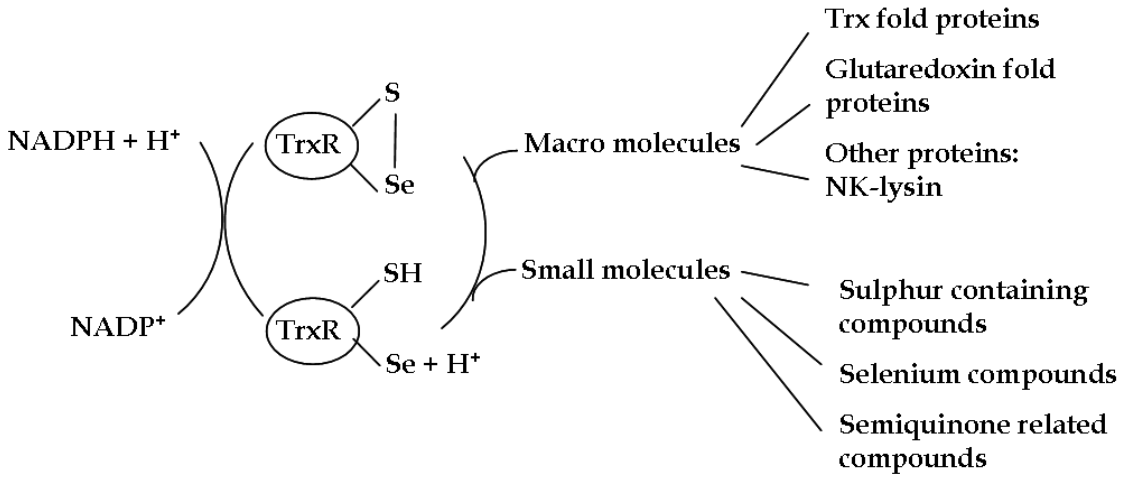

Figure 12: Electron transfer from NADPH via mammalian thioredoxin reductases to different substrates. Mammalian TrxRs have a wide substrate range. The substrates include generally macromolecules in which disulfide bonds play critical roles in the regulation of their functions, but also low molecular- weight compounds act as substrates for TrxRs.

The thioredoxin system plays a central role in the regulation of gene expression via redox control of transcription factors including NF-κB, Ref-1, AP-1, p53, glucocorticoid receptor, and apoptosis-regulating kinase (ASK1), thus indirectly regulating cellular activities such as cell proliferation, cell death, and immune-response activation (Rundlof and Arner, 2004). The redox sensitive Sec residue within TrxR has been suggested to act as a cellular redox sensor and regulator of cell signaling in response to elevated levels of reactive oxygen species (ROS) (Sun et al., 1999).

1.5.3. Iodothyronine deiodinases

The iodothyronine deiodinases family (DIOs) consists of three differentially distributed, Sec-containing oxidoreductases (DIO1, DIO2, DIO3) that catalyze the activation (DIO1 and DIO2) and inactivation (DIO3) of the thyroid hormones thyroxine (T4), 3,5,3′- triiodothyronine (T3), and reverse-3,5,3′-triiodothyronine (rT3) by removing distinct iodine moieties, as schematically indicated in (Bianco et al., 2002).

Thyroid hormone metabolism is dependent upon the combined actions of the three deiodinases and is regulated mainly through DIO2 stability in response to changes in

iodine supply, to cold exposure, and to changes in thyroid gland function (Gereben et al., 2000).

1.5.4. Selenoprotein P (Sel P)

Selenoprotein P is an abundant extracellular glycoprotein that is rich in selenocysteine. Sel P is secreted to the plasma by the liver in a glycosylated form; however its expression is detected in all tissues. It is the only characterized mammalian selenoprotein that contains more than one Sec residue (10 Sec residues). Moreover, it has been proven to be a central and multifunctional protein, which mediates Se transport, tissue-specific Se uptake and Se storage (Burk and Hill, 2005).
It has two domains with respect to selenium content. The *N*-terminal domain of the protein contains one selenocysteine residue in a UxxC redox motif. This domain also has a pH-sensitive heparin-binding site and two histidine-rich amino acid stretches. The smaller *C*-terminal domain contains nine selenocysteine and ten cysteine residues. Evidence supports functions of the protein in selenium homeostasis and oxidant defense (Burk and Hill, 2009).

1.5.5. Selenoprotein 15

Selenoprotein 15 (Sep15) was discovered in the late 1990s in human T-cells as a 15 kDa selenoprotein (Gladyshev et al., 1998b). The gene for Sep15 consists of five exons and four introns and is localized on chromosome 1p31 that is a locus often deleted or mutated in human cancers. The highest levels of Sep15 were found in human and mouse liver, kidney, prostate, brain and testes, but its level was reduced in hepatocarcinoma and a cancer prostate cell line (Kumaraswamy et al., 2000). Interestingly, two polymorphic sites occur in the human Sep15 gene at nucleotide positions 811 (C/T) and 1125 (A/G) in the 3'UTR and one of these, an A1125/G1125 polymorphism, is present in the Sep15 selenocysteine insertion sequence (SECIS) element (Gladyshev et al., 1998b). These polymorphisms manifest different responses to selenium supplementation and efficiency of Sec incorporation into protein (Hu et al., 2001). It was found to exist in a complex with UDP glucose glycoprotein glucosyltransferase (UGTR), a protein that is involved in the quality control of

protein folding (Parodi, 2000), and to be localized in the endoplasmic reticulum of mammalian cells (Korotkov et al., 2001).

1.5.6. Other selenoproteins

Selenoprotein H (Sel H) is a 14kDa, thioredoxin fold-like protein that contains a conserved Cys-X-X-Sec motif (X is any amino acid). Its expression is widely distributed throughout a variety of tissues and relatively high in early stages of embryonic development (Novoselov et al., 2007). Sel H is localized to the nucleus and over-expression studies suggest it is a redox-responsive DNA binding protein of the AT-hook family and functions in regulating expression levels of genes involved in *de novo* glutathione synthesis and phase II detoxification in response to redox status. Thus, SelH protected intracellular GSH and antioxidant levels and increased expression of key enzymes in GSH biosynthesis and in phase II detoxification (Panee et al., 2007).

Selenoprotein I (Sel I, hEPT1) was among several selenoproteins with no assigned function until a recent study found it to contain sequence homology to enzymes involved in phospholipid synthesis. Specifically, CDP-ethanolamine diacylglycerol ethanolamine-phosphotransferase (EPT) catalyzes the transfer of phosphoethanolamine from CDP-ethanolamine to diacylglycerol to produce phosphatidylethanolamine, and Sel I was identified as possessing a CDP-alcohol phosphatidyltransferase motif, a common motif conserved in phospholipid synthases (Horibata and Hirabayashi, 2007).

Selenoprotein K (Sel K) is a small (16 kDa) protein localized to the endoplasmic reticulum (ER) membrane (Lu et al., 2006) and some evidence suggests that it is associated with the plasma membrane (Kryukov et al., 2003).

Selenoprotein N (SelN) is established as an important protein in muscle physiology; mutation in SelN causes inherited muscular disease in humans (Schomburg, 2010).

Selenoprotein O (Sel O) is one of the selenoproteins that has remained enigmatic since identification of its sequence in the human genome several years ago. While human Sel O is predicted to consist of 669 amino acids with a calculated M.W. of 73.4 kDa, there is no information regarding its tissue distribution, subcellular location, or physiological role. The presence of a Cys-X-X-Sec motif is suggestive of a redox function, though experimental data are lacking (Kryukov et al., 2003).

SelR, also known as methionine-*R*-sulfoxide reductase B1 (MsrB1), is a member of the Msr family of proteins, which catalyze the reduction of oxidized methionine (Met) residues (methionine sulfoxides). Oxidation of Met occurs in response to an increase in ROS, which can lead to protein damage, and if unrepaired, to abrogated protein function (Kim and Gladyshev, 2004).

Selenophosphate-synthetase 2 (SPS2) is an enzyme involved in the biosynthesis of selenoproteins. Specifically, selenocysteyl-tRNA[Ser]Sec is aminoacylated by seryl-tRNA synthase and the seryl moiety is phosphorylated by PSTK as mentioned above to form *O*-Phosphoseryl- tRNA[Ser]Sec (Lacourciere, 1999).

Selenoprotein S is a transmembrane protein located in the ER and plasma membranes and is widely expressed in a variety of tissues (Ye et al., 2004). It has been suggested to participate in the removal of misfolded proteins from the ER lumen for degradation (Gao et al., 2004) and to protect cells from oxidative damage (Gao et al., 2007) and ER stress-induced apoptosis (Curran et al., 2005).

Selenoprotein T (Sel T) is ubiquitously expressed throughout embryonic development and adulthood in rat, and most likely localized to ER through a hydrophobic domain (Grumolato et al., 2008). Sel T is a member of a subfamily of selenoproteins (also including Sel W, Sel H, and Sel V) that share sequence similarity containing a thioredoxin-like fold and a conserved Cys-X-X-Sec motif (Dikiy et al., 2007).

Selenoprotein W is a small protein (9.5 kDa) with the Sec residue present in the N-terminal region as part of the –CXXU- redox motif. Within the cell, SelW is localized predominantly in the cytoplasm, and a small fraction is bound to the cell membrane. SelW binds glutathione with very high affinity, which in early studies suggested a potential antioxidant function (Yeh et al., 1995).

2. The health benefits of selenium

Improvement of human health by supplementation with selenium is suggested by the poor status in this element. There is evidence that selenium deficiency can have adverse consequences for disease susceptibility and the maintenance of optimal health. Low selenium status may contribute to the aetiology of the disease process but in some cases it may be an outcome of the condition itself and may exacerbate disease progression (Rayman, 2000). Selenium intakes in most parts of Europe are considerably lower than in the USA, soils being a poorer source of selenium. Plasma or serum selenium concentrations measured within the 1990s in a selected number of European locations are shown in Figure 13. The upper level of the bottom tertile in the Nutritional Prevention Cancer trial is marked on the figure and these locations fall well within that tertile. Consequently, it might be predicted that a repeat of the NPC trial in these European locations would show a large effect of treatment.

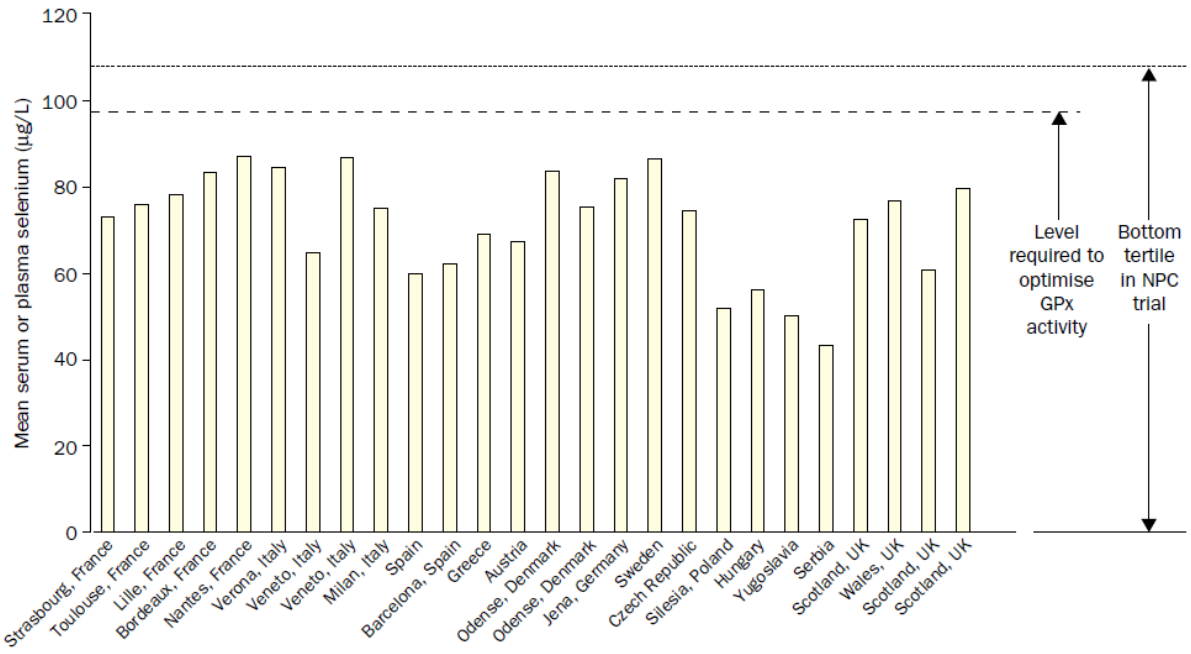

Figure 13: Mean concentrations, measured since 1990, of serum or plasma selenium in Europe compared with NPC trial levels and concentration required for optimal plasma GPx activity

Moderate selenium deficiency has been linked to many conditions, such as increased cancer and infection risk, male infertility, decrease in immune and thyroid function, and several neurologic conditions (Rayman, 2000). However, for some of these conditions, the evidence is rather scant, lacks consensus, and must be further demonstrated.

Diseases associated with selenium deficiency have been well documented for both animals and humans. Examples of Se-desifiency related diseases in animals include: hepatosis dietetic, a liver necrosis affecting pigs: exudative diathesis, a condition affecting poultry which results in edema of body tissues; and white disease, a nutritional muscular dystrophy which affects sheep and cattle (Combs, 2001). These deficiency disorders can result in death within days of symptom onset, resulting in obvious detrimental economic effects. Sub-clinical Se deficiencies have also been reported in livestock and are commonly associated with reduced growth and production, as well as a decrease in immune functions (Moir and Masters, 1979). Strategies in order to treat and prevent such deficiency diseases in livestock include the addition of Se to pastures and provision of salt licks containing sodium selenite.

2.1 Selenium and Cardiovascular diseases

Endemic selenium deficiency in humans was first reported in Keshan Province, in north-east of China, where the soil is exceptionally low in Se (intake, ≤10 μg/day) (Collipp and Chen, 1981). Keshan disease presented as a cardiomyopathy, primarly affecting children and women of child-bearing potential (Xu et al., 1997). General symptoms of Keshan's disease are arrhythmia, carsiac insufficiency, heart enlargement as loss of heart tissue. This disease was the first human disease related to selenium deficiency. Dietary supplementation with Se achieved a dramatic reduction in the incidence of this disease (Oster et al., 1983). Selenium may be protective against cardiovascular disease. On theoretical grounds, this hypothesis is supported by the ability of glutathione peroxidase to protect the tissue from the lipid peroxidation to reduce platelet aggregation (Levander et al., 1983). Moreover, GPx4 reduces hydroperoxides of phospholipids and cholesteryl esters associated with lipoproteins and may therefore reduce the accumulation of oxidised low-density lipoproteins in the artery wall (Koehler et al., 1988).

Selenium mainly provides a means of defense against the build-up of lipid peroxides and free radicals that damage all membranes and macromolecules including DNA.

2.2 Selenium and the Immune Response

Not all classes of antibodies are affected to the same extent by selenium deficiency and that differences in animal species, age, sex and antigens affect the degree to which antibody production responds to selenium supplementation (Arthur et al., 2003).

Numerous studies suggest that deficiency in selenium is accompanied by loss of immunocompetence, probably not connected with the fact that selenium is normally found in significant amounts in immune tissues such as liver, spleen, and lymph nodes. Both cell-mediated immunity and B-cell function can be impaired. Activated T cells show upregulated selenophosphate synthetase activity, directed towards the synthesis of selenocysteine, which shows the importance of selenoproteins to

activated T-cell function and the control of the immune response (Spallholz et al., 1990). At human nutritional doses, Se is essential for an optimum immune response and affects both the innate and acquired immune systems.

The principal mechanism stimulated by selenium can be summarized as follows: (i) detoxification of organic hydroperoxides and hydrogen peroxide; (ii) regulation of the balance of the activity in the eicosanoid synthesis pathways, leading to preferential synthesis of leukotrienes and prostacyclins over thromboanes and prostaglandins; (iii) down regulation of cytokines and adhesion molecules expression; (iv) up-regulation of on interleukin-2 receptor expression leading to enhanced activity of lymphocytes, natural killer and lymphokine activated killer cells (Hoffmann, 2007).

2.3 Selenium and neurodegenerative diseases

Selenium is retained within the brain even under conditions of dietary selenium deficiency, implying the potential importance of the trace element in neurological disorders (Behne et al., 1988).

Oxidative stress and generation of reactive oxygen species (ROS) are strongly implicated in a number of neuronal and neuromuscular disorders, including stroke and cerebrovascular disease, Alzheimer's disease (Sagara et al., 1998), Parkinson's disease (Dexter et al., 1989) familial amyotrophic lateral sclerosis, and Duchenne muscular dystrophy (Ragusa et al., 1997). Selenium is known to provide protection from ROS-induced cell damage, and the proposed mechanisms mainly invoke the functions of glutathione peroxidases (GPxs) and selenoprotein P (SelP).

Considerable evidence exists linking heavy metals to neurodegenerative diseases (Cornett et al., 1998). Selenium has long been known to function as an antidote to toxicity of heavy metals. Administration of selenium was reported to play a role in reducing the toxic effects of mercury as early as the 1970s (Kosta et al., 1975). Among the selenoproteins, SelP has been reported in several studies to possess metal-binding function. The GPxs might also detoxify heavy metals through their well-known function of eliminating peroxides.

When selenium is deficient in the diet, the brain shows high priority to conserve this element. In rats, prolonged selenium depletion over six generations led to a drastic decrease in the selenium concentrations in liver, skeletal muscle, and blood below 1% of normal levels, but the brain still contained a striking 60% of the concentration found in control animals (Kuhbacher et al., 2009). Early embryonic lethality resulted from knocking out the selenocysteyl-tRNA gene in mice, which led to the total disruption of selenoproteins synthesis (Bosl et al., 1997). In contrast, in rats fed a selenium-deficient diet for 16 generations, no increased mortality could be observed (Behne and Kyriakopoulos, 2001). This may indicate the presence of essential selenoproteins in the brain.

2.4 Selenium and cancer

Two major areas of research on the link between selenium and cancer have emerged. The first one is therapy, where progressing malignant cells have been reported to be more sensitive to selenium cytotoxicity compared to normal cells. The second one is prevention, where continuous low to moderate oral doses have been reported to prevent cancer. The extent of overlap between the two fields is still not evident. What complicates the role of selenium in cancer prevention is that selenium can acts both as an antioxidant and an oxidant depending on dose giving, form given, way given and properties of the receiving cells.

Solid evidence based on epidemiological studies conducted in the last 50 years show an inverse relationship between Se intake and cancer mortality. Thus the anticarcinogenic effect of Se against leukaemia and cancers of the colon, rectum, pancreas, breast ovaries, prostate, bladder, lung and skin seems clear under at least some conditions (Navarro-Alarcon and Cabrera-Vique, 2008).

The greatest consistency was noted for breast and prostate cancers (Trumbo, 2005). Silvera and Rohan also reported evidence to support an inverse relationship between Se exposure and prostate cancer risk, and possibly a reduction in risk with respect to lung cancer, although additional study is needed (Navarro-Alarcon and Cabrera-Vique, 2008).

The first observation of relationship between selenium intake and risk of cancer were done in US, when mortality due to lymphomas and cancers of the gastrointestinal tract, peritoneum, lung, and breast was observed to be lower for men and women residing in areas of the United States that have either moderate or high concentrations of Se in forage crops than for those residing in low-forage Se areas. Since many studies have shown that cancer patients present lower Se status than healthy controls. Several prospective epidemiological studies, in Finland, Netherland have shown relationships of Se status at entrance and occurrence of cancer during the follow-up (Combs, 2001). In regard of all the experimental and epidemiological data demonstrating a potential benefit of selenium in decreasing cancer risk, it seemed logical to develop supplementation trials to test this hypothesis. A first benefit was observed in China on the incidence of primary liver cancer that was significantly reduced by enriching flour with selenium or giving selenium pills to patients seropositive for hepatitis B virus (Yu et al., 1991). The Lixian study also realized in China, observed a reduction of 21% on stomach cancer but with a mixture of selenium, carotene and vitamin E (Blot et al., 1993).

The Nutritional Prevention of Cancer (NPC) trial (Clark et al., 1996) is the only randomized clinical trial to date to test the effect of selenium supplementation on cancer in a Western population. A total of 1312 patients (mean age, 63 years; range, 18-80 years) with a history of basal cell or squamous cell carcinomas of the skin were randomized from 1983 through 1991. The patients were treated for 4.5 years with 200 μg of yeast-enriched selenium or placebo and were followed for 7 years.

Selenium treatment did not protect against development of basal or squamous cell carcinomas of the skin. However, results from secondary end-point analyses supported the hypothesis that supplemental selenium may reduce the incidence of cancer incidence (Figure 14); in particular lung, prostate and colorectal.

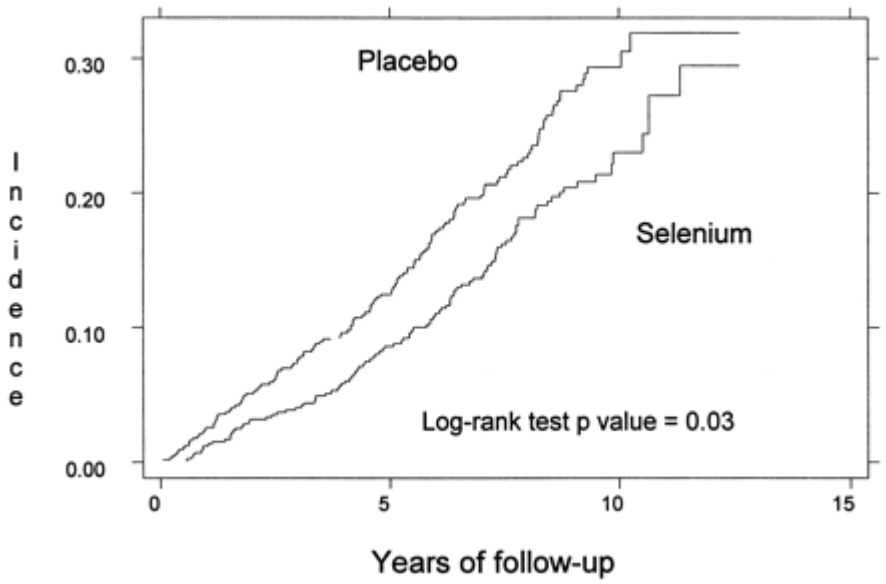

Figure 14: Cumulative incidence of total cancer in the NPC Trial by treatment group (Duffield-Lillico et al., 2002).

An additional paper by Clark et al. (Clark et al., 1998) showed that the effect of selenium supplementation on prostate cancer was accentuated among men in the lowest tertile of baseline plasma selenium level (Figure 15).

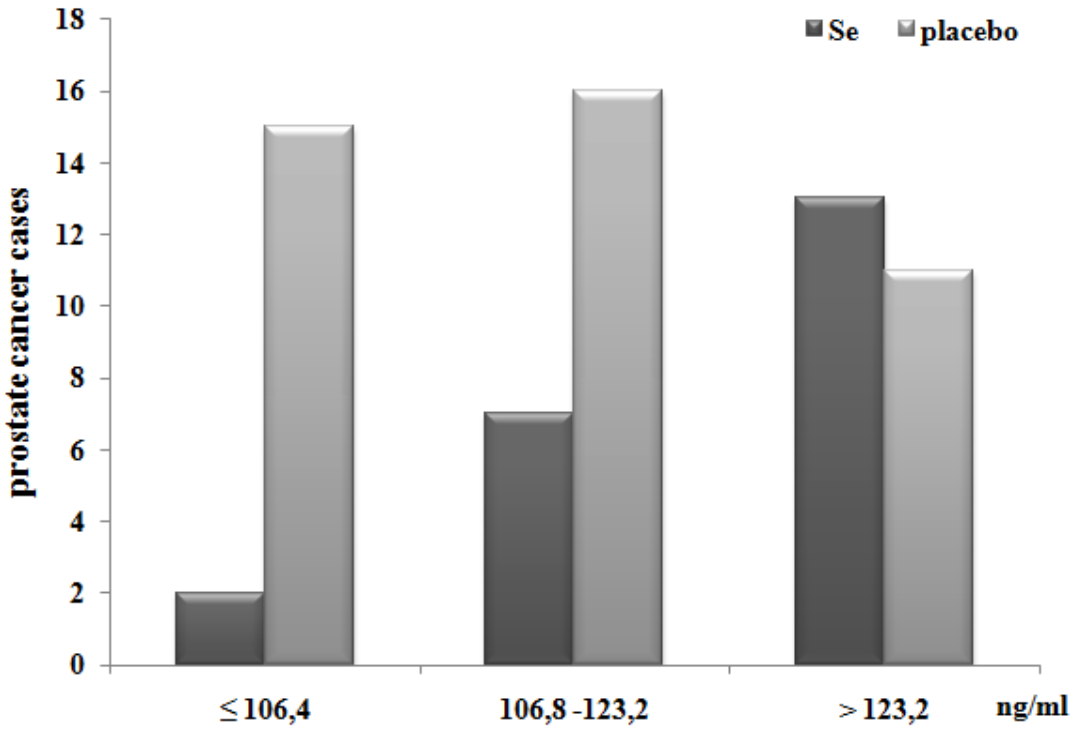

Figure 15: Protective effect of selenium supplementation was restricted to people with lower plasma selenium concentration before the supplementation

Duffield-Lillico et al. also showed that the protective effect of Se treatment against prostate cancer was significant only for subjects who entered the trial with relatively low plasma Se levels (Duffield-Lillico et al., 2003). Those entering with plasma Se below 106.4 ng/ml (1.35 nmol/l), that is, in the lowest tertile of that cohort, showed the strongest effect of Se treatment in reducing the risk of being diagnosed with prostate cancers over the subsequent years of follow-up. Subjects entering in the middle tertile of plasma Se, 106.8–123.2 ng/ml (1.37–1.58 nmol/l), showed a more modest, but still protective effect of Se treatment; but subjects entering in the highest tertile of plasma Se (4123.2 ng/ml or 41.58 nmol/l) showed no significant treatment effect.

The "Supplémentation en Vitamines et Minéraux Antioxydants" (SU.VI.MAX) study is a randomized, double-blind, placebo-controlled primary prevention trial. A total of 13 017 French adults (7876 women aged 35-60 years and 5141 men aged 45-60 years) were included. All participants took a single daily capsule of a combination of 120 mg of ascorbic acid, 30 mg of vitamin E, 6 mg of beta carotene, 100 µg of selenium, and 20 mg of zinc, or a placebo. Median follow-up time was 7.5 years. Only a significant interaction between sex and group effects on cancer incidence was found. Sex-stratified analysis showed a protective effect of antioxidants in men but not in women. After 7.5 years, low-dose antioxidant supplementation lowered total cancer incidence and all cause mortality in men but not in women. Supplementation may be effective in men only because of their lower baseline status of certain antioxidants (Hercberg et al., 2004).

Nevertheless, not all cancer prevention trials indicated reduced cancer risk by Se supplementation. SELECT, the Selenium and Vitamin E Cancer Prevention Trial, is based on the presumed antioxidant and anticancer properties of these agents that inhibit specific cellular processes in the development of cancer. SELECT is an intergroup phase III, randomized, double-blind, placebo-controlled, 200 µg/d of selenium and 400 mg/d of vitamin E alone and in combination in 32,400 healthy men with a digital rectal examination (DRE) not suspicious for cancer and a serum prostate specific antigen (PSA) ≤ 4 ng/ml (Klein et al., 2003). The large human prostate cancer prevention trial SELECT was stopped early in the seventh year in

October 2008 and the results indicated that there were no beneficial effects of Se against cancer incidence (Lippman et al., 2009). A lack of positive effect of Se supplementation on the prostate cancer incidence observed in this study was likely caused by the fact that different Se forms were used, selenomethionine compared to yeast-enriched selenium in the NPC trial. Se status was already optimal before supplementation and all selenoproteins would have been optimised. This trial did not test different formulations or doses of Se and there has not been any information as to whether Se might be effective among those with the lowest baseline Se levels (Ledesma et al., 2010). Moreover, Se status and genetic variation of tested individuals may represent additional reasons for negative results.

3. Mechanism of cancer prevention by selenium

Because cancer is not a single pathology, but a heterogeneous condition, it is unlikely that it can have a single cause or that an anticarcinogenic agent, such as selenium, acts trought a single mechanism relevant to all tumor types in which the element appears to be effective. Consequently, different hypotheses have been put forward to explain the role of selenium in cancer prevention, for each of which there is a reasonable amount of supporting evidence. Some of these hypotheses have implications for human health considerably wider than the area of cancer alone and reflect the key part that selenium plays in the whole of cell metabolism.

A number of mechanisms have been suggested to explain the anti-cancer effects of Se. Although there is fairly general acceptance that methyl selenol (CH_3SeH) is involved in the anti-cancer effects of Se at supra-nutritional doses, evidence is accruing, some from effects of functional selenoprotein polymorphisms, that the selenoenzymes do play a role, particularly at nutritional levels of intake (Brozmanova et al., 2010). Se in selenoproteins can reduce oxidative stress and limit

DNA damage which has been linked to cancer risk. Some of these anti-cancer processes or pathways will be more extensively discussed below.

3.1 Role of selenoproteins

A link between selenoproteins and cancer risk was originally based on the antioxidant activities of some selenoproteins, such as enzymes of glutathione peroxidase (Gpx) family and selenoprotein P (SelP) which provide antioxidant protection against reactive oxygen species (ROS)-driven cancer initiation and promotion.

Excessive ROS generation ($O_2^{\cdot-}$,$^1O_2$, $^{\cdot}OH$ and H_2O_2) or inadequate antioxidant protection induces persistent oxidative stress that contributes to the expression of malignant phenotype of cancer cells by genetic changes in oncogenes and/or tumor suppressor genes (Valko et al., 2007).

However, moderate perturbations of redox homeostasis usually initiate a signaling response. Such redox signaling is a well-recognized stress response with a variety of downstream effects, including upregulation of protective and repair enzymes. The enzymatic systems include a set of gene products such as superoxide dismutases, catalases, thioredoxin (Trx) and glutathione peroxidases (GPx) system (Margis et al., 2008).

An important feature of the antioxidant network is that its components act in synergy to destroy reactive oxygen species. Such synergistic interactions are reinforced by mutual protections of antioxidant enzymes. For example, SODs protect Se-GPx and catalase from inactivation by superoxide, while Se- GPx and/or catalase protect SODs from inactivation by hydroperoxides. Moreover, vitamins promote the action of several enzymes to make them work more efficiently. In this role they are called coenzymes. Much research has been carried out to determine minimal amounts of vitamins that are needed to perform their necessary action.

Actually, the Trx and GSH systems are also involved in a variety of redox-dependent pathways such as providing reducing equivalents for ribonucleotide reductase (the first step in DNA biosynthesis) and peptide methionine sulfoxide reductase, an antioxidant defense and regulation of the cellular redox state. In addition, the Trx

and GSH systems regulate activities of various transcription factors, kinases, and phosphatases, and they are implicated in the redox control of cell growth and death, transcription, cell signaling, and other processes (Lander, 1997).

Reduction of one or more selenoproteins levels increases the likelihood of cancer development. Three possible pathways could be involved to account for this decrease: 1) reduced dietary selenium intake; 2) genetic polymorphisms that result in an increased selenium requirement for baseline levels of protection; and 3) allelic loss of 1 of 2 gene copies during tumor development.

Genetic data indicating functional polymorphisms in the genes for several selenoproteins, such as GPx or Sep15, and more significantly, the association of allelic variants with cancer risk, are a compelling argument for the involvement of those genes, and by logical extension, the mediators of the benefits of selenium (Diwadkar-Navsariwala et al., 2006).

Deletion of the gene for selenoprotein P in mouse models alters the distribution of selenium in body tissues suggesting that selenoprotein P is required for selenium transport (Hill et al., 2003). While the human selenoprotein P gene (SEPP1) is abundantly expressed in normal colon mucosa, there is a significant reduction or loss of SEPP1 mRNA expression in colon cancers (Al-Taie et al., 2004). Expression of SEPP1 is also dramatically reduced in a subset of human prostate tumors, mouse tumors and in the androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cell lines (Calvo et al., 2002). Homozygosity for the Ala234 allele of the SEPP1 Ala234Thr SNP (rs3877899) is associated with a lower concentration of plasma selenoprotein P in men, affecting the concentration and/or activity of other selenoproteins, notably of thioredoxin reductase 1 (TrxR1) and some of the antioxidant glutathione peroxidases (GPx) (Cooper et al., 2008).

The loss of heterozygosity in GPx1 was observed in DNA from breast, head and neck tumors, implicating a possible involvement of Gpx1 in the development of these types of cancer (Hu and Diamond, 2003). The GPx1 gene polymorphism at position 198 causing substitution of proline for leucine is associated with an increase of bladder, lung and possibly breast cancers (Ichimura et al., 2004). The identity of the

amino acid at codon 198 (proline or leucine) has functional consequences with regard to the level of enzyme activity in response to increasing levels of selenium provided to cells in culture (Hu and Diamond, 2003). In contrast, GPx1 Pro198Leu genotype was determined in 82 prostate cancer cases and 123 control individuals and an overall protective effect of the variant Leu allele of the GPx1 polymorphism was found on the prostate cancer risk (Hu and Diamond, 2003).
The allelic loss of Sep15 may be involved in breast cancer progression (Jablonska et al., 2008).

Prevention of cancer by selenium may be also related to the Trx system through its general antioxidant protective roles or through specific pathways (and likely a combination of the two). It is well known that increased oxidative stress may have mutagenic consequences and the Trx system may contribute to a general protection from such events. Other more direct links between selenium and the Trx system in the protection of cells from cancer development may possibly be found in the dependence of p53 on a functional Trx system (Ueno et al., 1999). This, in turn, may possibly relate to the switch of p53 to a protective DNA repair system by selenomethionine (Gudkov, 2002), provided that the selenomethionine in that case increased TrxR expression and thus the p53-supportive activity of the Trx system.

3.2 Reduction of DNA damage

Evidence that Se can reduce DNA damage comes from studies in dogs and human. In a canine model of prostate cancer forty-nine elderly male beagle dogs, physiologically equivalent to 62–69-year-old men and similarly subject to prostate cancer, received nutritionally-adequate or supranutritional levels of dietary Se as selenomethionine or Se-enriched yeast for 7 months. DNA damage in the prostate was measured by the alkaline comet assay while Se was measured in toenails. The percentage of prostate cells with extensive DNA damage was found to fall with increased Se exposure up to a level of 0.8–0.9 mg/g, as measured in dog toenails.

Damage began to rise at >1.0 mg/g toenails, demonstrating the typical 'U'-shaped response (Figure 16) to a nutrient that is toxic at high levels (Waters et al., 2005).

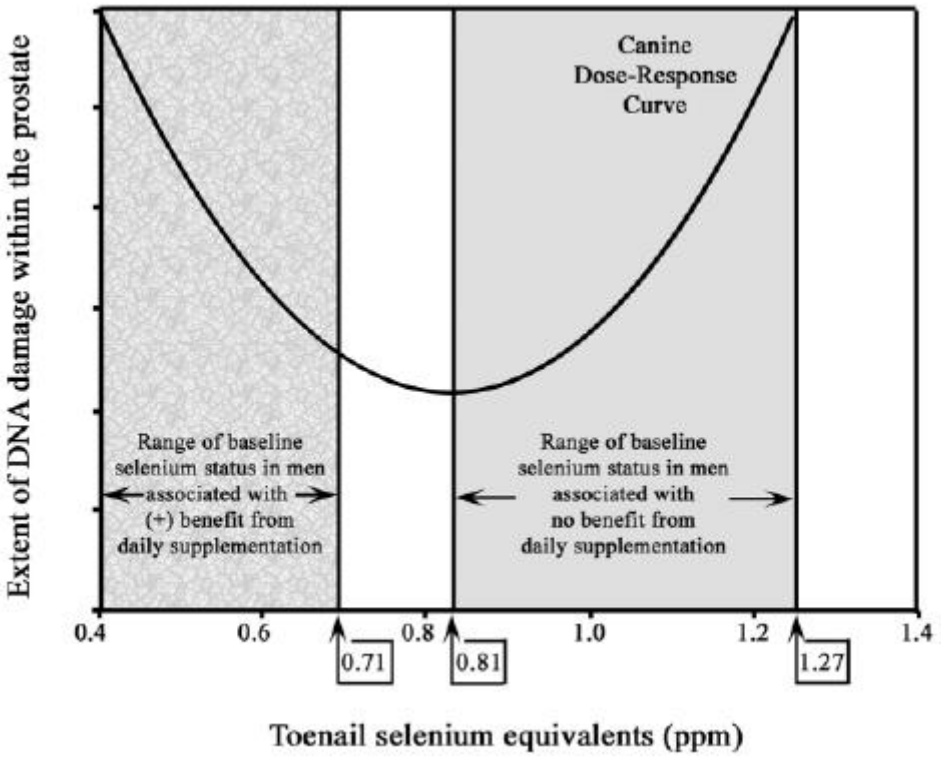

Figure 16: A U-shaped dose--response curve defines the relationship between selenium and genotoxic stress in prostate. Canine dose--response curve explains the effect of baseline selenium status on human prostate cancer risk reduction in the Nutritional Prevention of Cancer Trial.

In a New Zealand study of men aged 50–75 years at risk of prostate cancer, the comet assay was reported to show a significant inverse relationship with overall accumulated DNA damage in blood leucocytes from subjects with serum Se levels below the mean (Karunasinghe et al., 2004).

3.3 Inhibition of DNA adduct formation

Microarray data have indicated that Se, independent of its form and target organ examined, alters the expression of a number of genes in a manner that may account for cancer prevention. Se can up-regulate genes related to phase II detoxification enzymes, certain genes encoding Se-binding proteins and selected apoptotic genes,

while down-regulates those related to phase I activating enzymes and cell proliferation (El-Bayoumy and Sinha, 2005). The phase I activating enzymes include members of the cytochrome P450 system responsible for converting chemical carcinogens to reactive adducts that can attack DNA. Thus, Se can act protectively in the initiation phase of carcinogenesis by decreasing the formation of active reactive forms of carcinogens (el-Bayoumy et al., 1992).

3.4 Effect on DNA repair

Importantly, an enhancement of DNA repair capacity may represent an alternative mechanism by which Se exerts its anticancer properties. Until now, very few studies have explored enhancement of DNA repair as a possible selenium mechanism of action.

It was shown that Se in the form of SM induces a DNA repair response in normal human fibroblasts *in vitro* and protects cells from DNA damage induced by ultraviolet light (UV, 254 nm) and H_2O_2 exposures (Seo et al., 2002b). The Comet assay employed UV-radiation as the DNA-damaging agent, thereby implicating the nucleotide excision repair (NER) pathway, which is responsible for repair of UV-damage as well as bulky carcinogen adducts. They considered that UV-radiation also causes release of reactive oxygen species (ROS) by damaged mitochondria (Gniadecki et al., 2000). Because thioredoxin reductase and glutathione peroxidase are selenoenzymes, that can directly scavenge free radicals, SM induction of these enzymatic activities may also play a role in protection from DNA damage.

Yet, the Host Cell Reactivation (HCR) assay employed in this study involved only a UVC-damaged reporter plasmid (Seo et al., 2002b). Seo at al. also showed the enhancement of PCNA-dependent repair complex formation. This finding argues for an inducible DNA repair response, coincident with induction of some DNA repair proteins. Importantly, PCNA-dependent complex formation is required not only for NER, but also for repair of base damage by the base excision repair (BER) pathway (Karmakar et al., 2001).

Seo et al. also demonstrated that selenium concentration is a determinant of basal p53 activity, and that protection from DNA damage by SM is p53-dependent.

Selenomethionine can activate p53 with redox mechanism independent of DNA damage that requires the redox factor Ref1. With Selenium concentrations used in that study, 20 μM for 15 h, p53-dependent DNA repair was activated. They used a 20kDa carboxyl-terminal fragment of p53 (ct-p53) to define further the p53 residue(s) responsive to selenium treatment. It was clear showed that ct-p53 was strongly reduced as a response to selenium, thereby implicating Cys-275 and/or Cys-277, although it is not exclude that additional cysteines play a role in full-length p53 (Seo et al., 2002a). Importantly, Cys-277 constitutes part of the active (sequence-specific DNA binding) site (Rainwater et al., 1995), consistent with the results showing enhanced sequence-specific binding in the presence of SM.

More recently, selenomethionine has been reported to stimulate repair of bleomycin-induced DNA damage in human leukocytes *in vitro,* and also on repair and persistence of this damage, when applied before and simultaneously with bleomycin (Laffon et al., 2010). These data support the previously proposed mechanisms of chemoprotection of SM as related to its ability to interfere with DNA repair pathways (Santos and Takahashi, 2008).

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4. DNA damage

The genetic information required for all life is encoded DNA, which is constantly being damaged as a consequence of normal cellular metabolism and by exposure to genotoxic agents. DNA damage left unrepaired results in mutations that can alter the genetic information stored in the DNA. Cells have consequently evolved complex mechanisms to combat mutation of their genetic material. Defects in the cellular response to DNA damage can result in genomic instability, a hallmark of cancer cells. Because human somatic cells are diploid, mutant genes are most often recessive when they arise in a somatic cell, and they usually do not initially express because of the presence of the wild-type copy of the homologous gene. However, recessive

mutations can express when there is a loss of heterozygosity (LOH). This condition may arise in a number of ways including mutation in the homologous gene, occurrence of aneuploidy (loss of a chromosome) for the homologous chromosome, or by recombination with the homologous chromosome.

An understanding of the chemistry of DNA damaging agents and the lesions they cause is necessary before one can seek out and study specific repair pathways. One of the consequences of having a DNA-based genome is that all cells suffer from DNA damage be it from endogenously or exogenously derived sources.

4.1 Endogenous DNA damage

4.1.1 Instability of DNA

Cellular genomes undergo extensive damage from the intracellular environment through hydrolysis and exposure to reactive metabolites that cause oxidation and alkylation of DNA. Apurinic/apyrimidinic (AP) sites, or abasic sites, probably represent the most frequent type of endogenous damage. AP sites occur spontaneously by hydrolytic loss of purine bases at a frequency of 10 000 per human cell per day (Lindahl, 1993). The exocyclic amino groups of some of the bases in DNA can be spontaneously substituted by water in reactions that are dependent on temperature and pH. An important hydrolysis reaction is deamination of cytosine and adenine to uracil and hypoxanthine, respectively. Both derivatives have miscoding properties and may produce mutations unless they are removed (Figure 17).

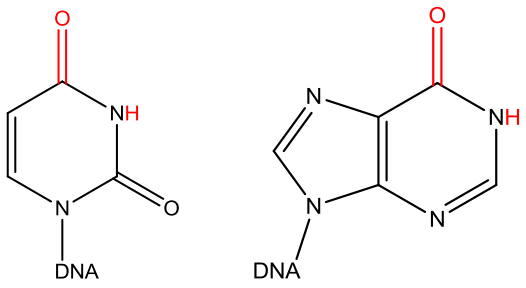

Figure 17: Chemical structures of Uracil and Hypoxanthine

Several enzymes, such as S-adenosylmethionine (SAM)-dependent methyltransferases could promote uncontrolled methylation; and other one of such as the activation-induced deaminase favorizes deamination (Lutz, 1990).

Another type of endogenous damage is the appearance of mispaired bases upon replication. The rate of mispairing can be significantly affected by cellular metabolism, chemical alterations of the bases, and by the presence of base analogues. A transient rearrangement of bonding among the bases (tautomeric shift) can occur during normal cellular metabolism. Such a rearrangement results in the production of a structural isomer of a base. These tautomers enhance misapiring.

4.1.2 Normal oxidative cellular metabolism

Oxygen is a critical part of life for higher organisms, except plants and algae. It is necessary for aerobic respiration which provides the greatest amount of cellular energy for most cells. Redox reactions of the respiratory chain ultimately use electrons to reduce oxygen to water. Under normal metabolic conditions 2–5% of the O_2 consumed by mitochondria is converted to reactive oxygen species (ROS). They are released during cellular respiration, process of biosynthesis and biodegradation, biotransformation of xenobiotics and phagocyte activation. There are about 60 enzymatic reactions that use O_2 as a substrate and release ROS. Oxidative stress is defined as a persistent imbalance between antioxidants and pro-oxidants in favor of the latter, resulting in (often) irreversible cellular damages.Within the mitochondria, oxygen free radicals are produced at complex I and III of the electron transport chain (Bergamini et al., 2004).

$$O_2 \xrightarrow{e^-} O_2^{\bullet -} \xrightarrow{2H^+ + e^-} H_2O_2 \xrightarrow{e^-} OH^{\bullet} \xrightarrow{H^+ + e^-} H_2O$$

Oxygen radicals react readily with metals, oxidants, and reductants yielding a gamut of reactive species. The most common ROS produced within the cell are superoxide anions ($O_2^{\bullet -}$). By itself, superoxide is only moderately reactive, but it is readily reduced into molecules that are highly reactive. Two molecules of superoxide

spontaneously convert to H_2O_2 and molecular oxygen, but this reaction is accelerated by superoxide dismutase (SOD).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\,H^+ \rightarrow H_2O_2$$

Another form of ROS produced in cells is hydroxyl radical ($^{\bullet}OH$). This species is formed when H_2O_2 oxidizes a transition metal (Fenton reaction) or when H_2O_2 reacts with superoxide (Haber-Weiss reaction).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$
$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH$$

Substantial evidence has been presented that H_2O_2 and $O_2^{\bullet-}$ do not react directly with DNA. Instead, the primary source of DNA damage caused by ROS seems to result from $^{\bullet}OH$. The hydroxyl radical can abstract hydrogen atoms from all five carbon atoms of 2'-deoxyribose resulting in base loss and/or strand breakage (Powell et al., 2005).

Hydroxyl radicals also react with bases (Figure 18). Attack on pyrimidines occurs at the C5-C6 double bond of thymine and cytosine and the 5-methyl group of thymine. $^{\bullet}OH$ adds to purines giving rise to C4-OH-, C5-OH-, and C8-OH adduct radicals. One-electron oxidation and one-electron reduction of C8-OH-adduct radicals give rise to 8-hydroxypurines (8oxoA and 8oxoG) and formamidopyrimidines (FapyA and FapyG), respectively.

8oxoG is one of the most abundant DNA oxidative lesions. This adduct is a mutagenic lesion that preferentially pairs with adenine rather than cytosine resulting in G:C to T:A transversions following replication.

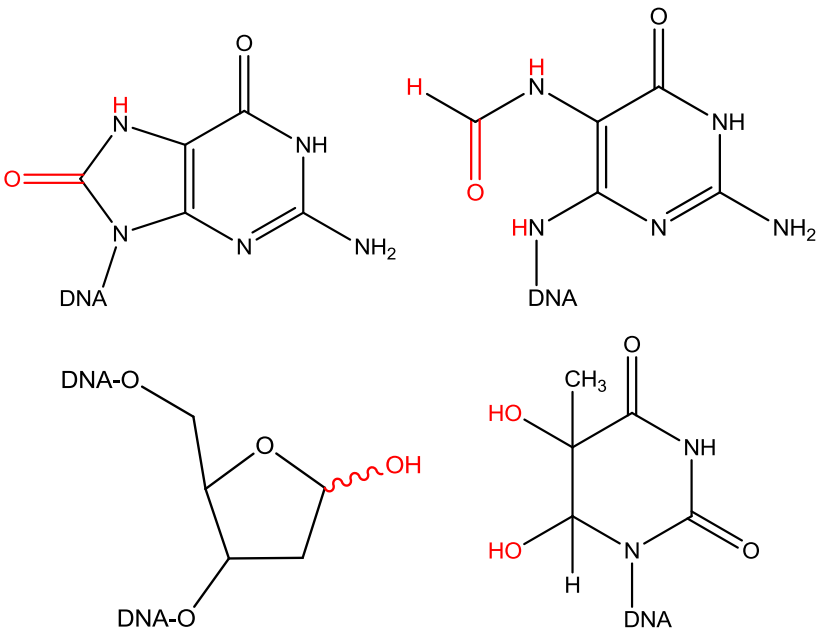

Figure 18: Chemical structures of DNA oxidative lesions respectively 8-oxoGua, FapyG, abasic site and thymine glycol.

4.2 Exogenous DNA damaging agents

4.2.1 UV irradiation

Ultraviolet (UV) radiation encompasses the radiation spectrum from 100-400 nm, and is divided into UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (100-280 nm). UV exposure at the earth's surface is only in the UV-A and B range. Yet numerous studies used UVC, the wavelength of which is close to the absorption maximum of DNA (267 nm).

Upon exposure to UV, the bases in the DNA strongly absorb UVC and UVB photon that leads to rearrangements of the chemical bonds. The first type of UV damage that was extensively studied was the cyclobutane pyrimidine dimer (CPDs) (Beukers et al., 2008). In this photoproduct, the double bonds of two adjacent pyrimidines fuse into a cyclobutane ring. Another type of dimer is produced when the C6 of one pyrimidine is linked to the C4 of an adjacent pyrinidine, and is referred as to pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs) (Cadet et al., 1997).

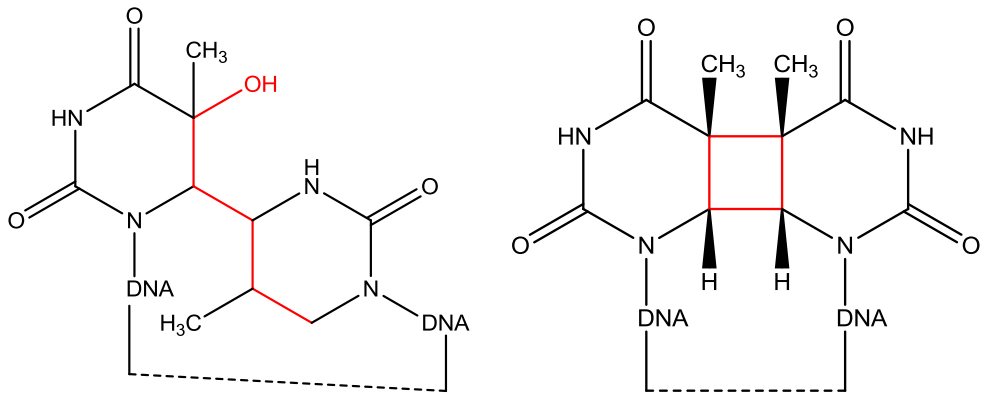

Figure 19: Chemical structures of 6-4 PD [T(6-4)T] and CPD [T<>T]

In addition to photoproducts arising from direct excitation in the UVC and UVB ranges, photosensitized reactions also play a major role in cells exposed to UVA and visible light. After absorption of photons by chromophores other than DNA, electron can be abstracted from DNA (type I photosensitization), or energy transferred to molecular oxygen, with formation of singlet oxygen in turn damaging the DNA molecule (type II photosensitization). The most widely studied photosensitization reactions in DNA involve oxidation processes which generate strand breaks and mostly 8-oxoGua (see 4.1.2). Although considered as typical UVB-induced DNA photoproducts, cyclobutane pyrimidine dimers are also involved in the genotoxicity of UVA. Indeed, UVA-induced cyclobutane pyrimidine dimers are formed *via* a direct photochemical mechanism, without mediation of a cellular photosensitiser (Mouret et al., 2010).

4.2.2 Alkylating agents

An important class of DNA reactive genotoxins is alkylating agents (Hensley et al., 1999). Human exposure to alkylating agents is frequent in daily life through occupational sources, medical treatments (Drablos et al., 2004), and several endogenous sources, including nitrosation of compounds in food and tobacco smoke into agents that alkylate DNA (Jenkins et al., 2005).

Alkylating agents can be classified as monofunctional or bifunctional (i.e., single or double reactive groups), and they are electrophilic compounds with an affinity for

nucleophilic centers in organic macromolecules. Humans are most frequently exposed to monofunctional alkylating agents. Four monofunctional alkylating agents were principally knew: methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), methylnitrosourea (MNU) and ethylnitrosourea (ENU) (Doak et al., 2007). MMS compound has been used as a solvent, insecticide, and chemotherapeutic agent, but due to its strong carcinogenic potential, it is now primarily used as model alkylating agent. MMS have high s values (Swain Scott constant used as standard measure of the reactivity of alkylating agents), thus react predominantly with ring N atoms, and consequently is potent mutagen due to mispairing during replication or the production of abasic sites and single-strand DNA breaks (Gocke et al., 2009).

The ring nitrogens on the DNA bases are more nucleophilic and therefore more susceptible to alkylation than are the oxygens, with N^7 of guanine and N^3 of adenine being the most reactive. Base alkylation of DNA can either be innocuous, mutagenic, or lethal to the cell.

The predominant adduct in double-stranded DNA resulting from MMS is N^7MeG, which comprises 84% of the DNA alkylation products, followed by N^3MeA (8.5%), N^3MeG (1.2%) and O^6MeG (0.3%) (Nikolova et al., 2010).

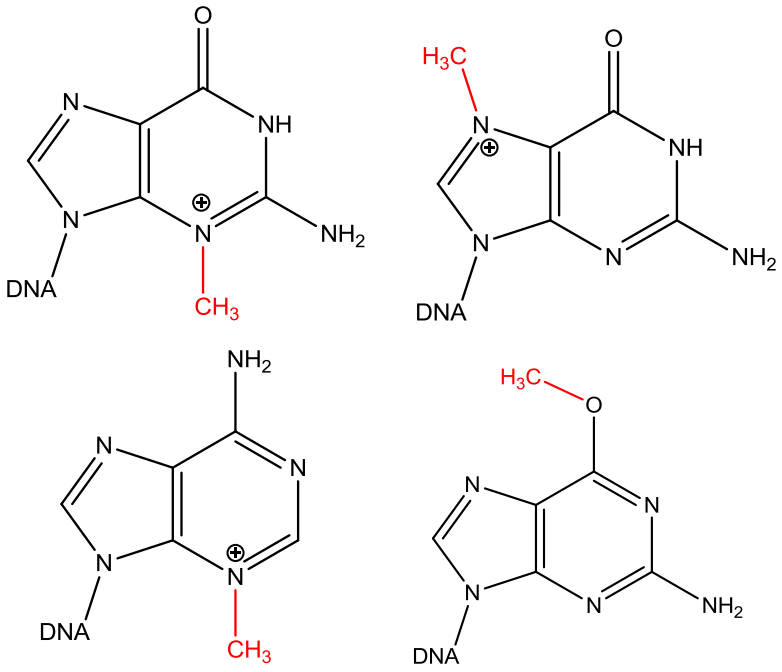

Figure 20: Chemical structures of several alkylating DNA damage. 3MeG, 7MeG, 3MeA and O^6MeG respectively

The main adduct, N^7MeG, does not block replication and is not miscoding, but its depurination produces an apurinic (AP) site, which if not repaired can be mutagenic and cytotoxic. In addition to its role as a source of AP sites, 7MeG can manifest toxic and mutation properties by converting to its imidazole ring-opened form, called Fapy-7MeG (O'Connor et al., 1988).

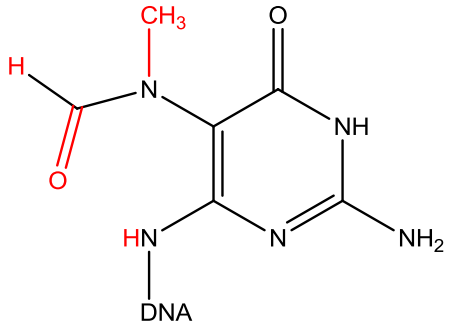

Figure 21: Chemical structure of Fapy-7MeG

3MeA is not particularly mutagenic; it is a cytotoxic DNA lesion by virtue of its ability to block replication or to generated AP sites. 3MeG is though to block replication in the same way as 3MeA does, but it is formed in DNA at a 15-fold lower level. Methylation of DNA at O^6 of guanine (O^6MeG) is a mutagenic event; this lesion causes G → A transitions (Shrivastav et al., 2010).

4.2.3 Ionizing radiation

DNA damage from ionizing radiation can occur from the direct deposition of energy to DNA, as well as indirectly through the interaction of ROS with DNA. The majority of DNA damage caused by ionizing radiation appears to be formed by reactive $^{\bullet}$OH, hence ionizing radiation causes a large amount of oxidative DNA damage. A peculiarity is the co-localization of damage along the track of the radiation. A typical example of RI-induced specific lesion is DSBs.

4.2.4 Chemotherapeutic compounds

Modern chemotherapy employs a wide range of efficient cytostatic agents, such as cisplatin and bleomycin. These molecules are active by damaging DNA and thereby preferentially killing fast dividing tumor cells. However, dangerous side effects sometimes hamper the therapy and may lead to serious or even fatal organ dysfunctions.

The N7 atoms of guanine and adenine are the main binding sites for platinum complexes in double-stranded DNA. The interaction between cisplatin and DNA can result in mono- and bifunctional adducts, as well as DNA–protein crosslinks. The bifunctional adducts, which can take the form of intra- or interstrand crosslinks, may cause major local distortions of DNA structure, involving both bending and unwinding of the double helix (Wozniak and Blasiak, 2002).

The antitumor antibiotic, bleomycin, causes oxidation at C4′ position of 2′-deoxyribose exclusively in single- and double-stranded DNA. It does not only form ROS but it can also act as an intercalating agent thanks to its bithiazole structural moiety (Kaiserova et al., 2006). Bleomycin thus induces sequence-specific single and double strand breaks in DNA by radical-based mechanisms. Bleomycin binds iron and oxygen thus forming an activated complex capable of releasing damaging oxidants in close proximity to DNA.

5. DNA repair

The consequences of DNA lesions include mutations, genetic recombination, and the inhibition or alteration of cellular processes such as replication and transcription. These direct effects can lead in turn to many indirect effects including chromosomal aberrations, tumorigenesis, evolution, apoptosis, and/or necrosis.

The primary mechanism by which organisms maintain their genomic functions in the face of these threats is by removing the lesions from the DNA and restoring the genomic integrity, a process known as DNA repair. Experimental studies in a variety of species have documented an incredible diversity of repair pathways. Fortunately, the comparison of repair pathways is simplified by the fact that all repair pathways can be placed into one of three classes based on its general mechanism of action: direct repair (Eker et al., 2009), recombinational repair (Thompson and Schild, 2002), and excision repair.

In direct repair, alterations in the structure of DNA are simply reversed. Examples include photo-reactivation, alkyltransfer, and DNA ligation. In recombinational repair, sections of altered or damaged DNA are corrected by homologous recombination with undamaged templates (Camerini-Otero and Hsieh, 1995). Thus, there is a great deal of overlap between the pathways involved in general recombination and those involved in recombinational repair.

Finally, in excision repair, a section of one strand of the DNA double-helix containing the modified base is first excised, then the other strand is used as a template to correctly re-synthesize the removed section, and finally the patch is ligated into place (Sancar, 1996). Figure 22 shows a summary of several DNA damaging agents, the associated DNA lesions induced and the different DNA repair systems involved.

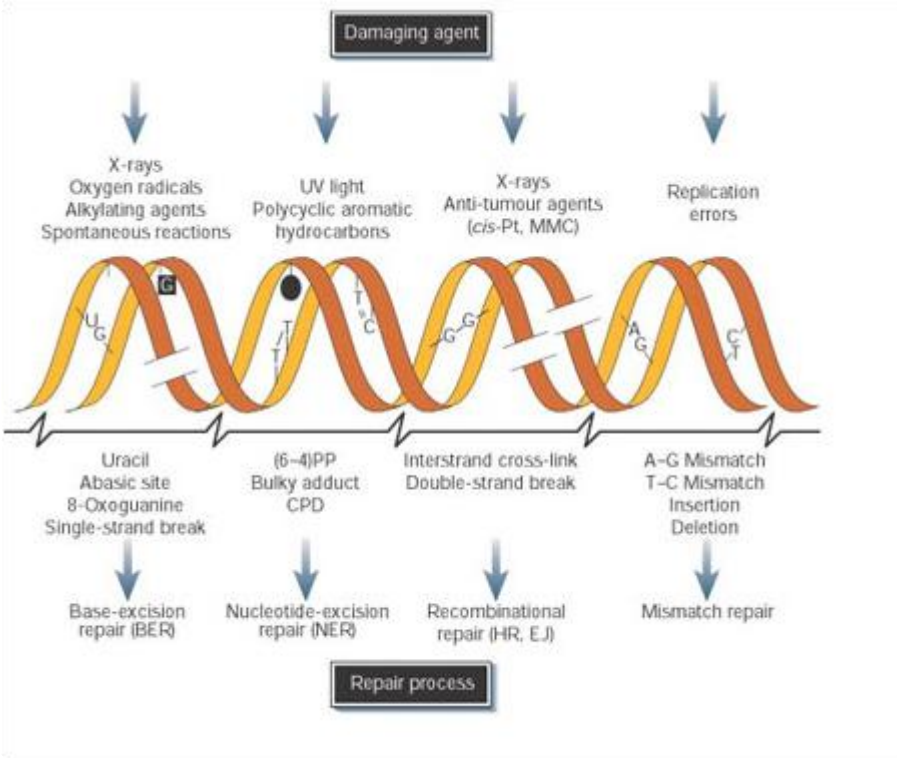

Figure 22: DNA damage and repair mechanisms summary adapted from (Hoeijmakers, 2001). Common DNA damaging agents (top), examples of DNA lesions induced by these agents (middle), and the most relevant DNA repair mechanism responsible for the removal of the lesions (bottom).

5.1 Base Excision Repair (BER)

BER is the main guardian against damage due to cellular metabolism, including that resulting from ROS, methylation, deamination and hydroxylation. BER pathway can be divided into five sequential steps: (1) excision of a modified or altered base; (2) cleavage of resulting AP site; (3) processing of the blocking termini at the break; (4) repair synthesis; and (5) ligation of broken strands (Friedberg, 2008).

The first BER step is catalyzed by lesion-specific DNA glycosylases that recognize the damaged nucleotide and excise the modified bases by cleavage of the N-glycosylic bond. DNA glycosylases are classified as mono- or bifunctional based on their reaction mechanism. Monofunctional glycosylases have only glycosylase activity, whereas bifunctional ones possess also AP lyase activity that permits them to cut the phosphodiester bond of DNA, creating a single-strand break without the need for an AP endonuclease.

Enzyme	Substrate
Uracil *N*-glycosylase family, monofunctional,	
MYH	**A:8oxo-Gua**
MPG	**3Me-A, hypoxanthine**
SMUG	**Uracil (+ 5-hydroxycytosine, 5-hydroxymethyluracil)**
TDG	**T:G mispair**
UNG	**Uracil**
Nth Family, bifunctional (β-elimination)	
NHT1	**Thymine and uracil glycols, 5-formyluracil, FapyG and FapyA**
OGG1	**8-oxoGua, FapyG**
Neil Family, bifunctional (βδ-elimination)	
NEIL1 (in ssDNA)	**FapyAde, FapyGua, Thymine glycols, 5-OHUra**
NEIL 2 (in ssDNA)	**Hydantoin, 5-OHUra**

Table 2: The main *N*-glycosylases involved in the repair of oxidized bases

The monofunctional DNA glycosylases attack the anomeric carbon N-C1′ of the damaged base to generate an AP site. The abasic site (which exists as equilibrium between an open aldehydic and a closed ring furanose form) is incised by an AP endonuclease or an AP lyase 5′ or 3′ of the base-less nucleotide respectively. Next, 5′ or 3′ phosphodiesterase activities remove the remaining sugar-phosphate at the DNA termini. The resulting gap is filled by a DNA polymerase and the strand is sealed by a DNA ligase.

Human enzymes of this type include methylpurine-DNA glycosylases (MPG, APNG and ANPG), uracil-DNA glycosylases (UNG), single-strand selective monofunctional uracil-DNA glycosylases (SMUG1), thymine DNA glycosylases (TDG), and MYH.
The bifunctional DNA glycosylases have an associated AP lyase activity that incises the strand 3′ of the abasic site. They use an activated amine moiety as a nucleophile for substitution of the damaged base, which leads to the formation of a Schiff base intermediate. The AP lyase activity then eliminates the phosphate group at the end 3′ of the nucleotide lesion. The remaining 3′ unsaturated abasic fragment is a substrate for AP endonucleases, and their action leads to a single-nucleotide gap that is filled by DNA polymerases. This type of glycosylases can be divided into two classes, depending on their mode of action in processing the AP site. One class, as exemplified by NTH1 and OGG1, cleaves the AP site through β-elimination, yielding a 3′-phospho-αγ-unsaturated aldehyde. While enzymes in the other class, such as endonuclease NEIL, carry out the so-called β,δ-elmination by further cleaving the deoxyribose residue, leaving a 3′-phosphate at the strand break (Zharkov, 2008).

Biochemical evidence has revealed at least two major subpathways in BER, the short-patch (single nucleotide) and the long-patch (more than one nucleotide), differing in their repair patch sizes and some participating proteins.
In the short-patch BER, the gap is filled through template-directed synthesis by POLβ which can also use its dRpase activity to remove any remaining 5′-dRp moiety at the strand break. The nick is sealed by the complex of DNA ligase 3 (LIG3) and X-ray cross-complementation protein 1 (XRCC1).
In the long-patch BER, POL δ and ε as well as POLβ can catalyze the repair patch synthesis. Together with proliferating cellular nuclear antigen (PCNA) and replication factor C (RFC), a polymerase displaces the 5′-dRp containing strand and synthesizes a segment of DNA 2-12 nucleotides long. The resulting flap-like structure is removed by the structure- specific flap endonuclease 1 (FEN1) and the nick sealed by LIG1 (Sattler et al., 2003).

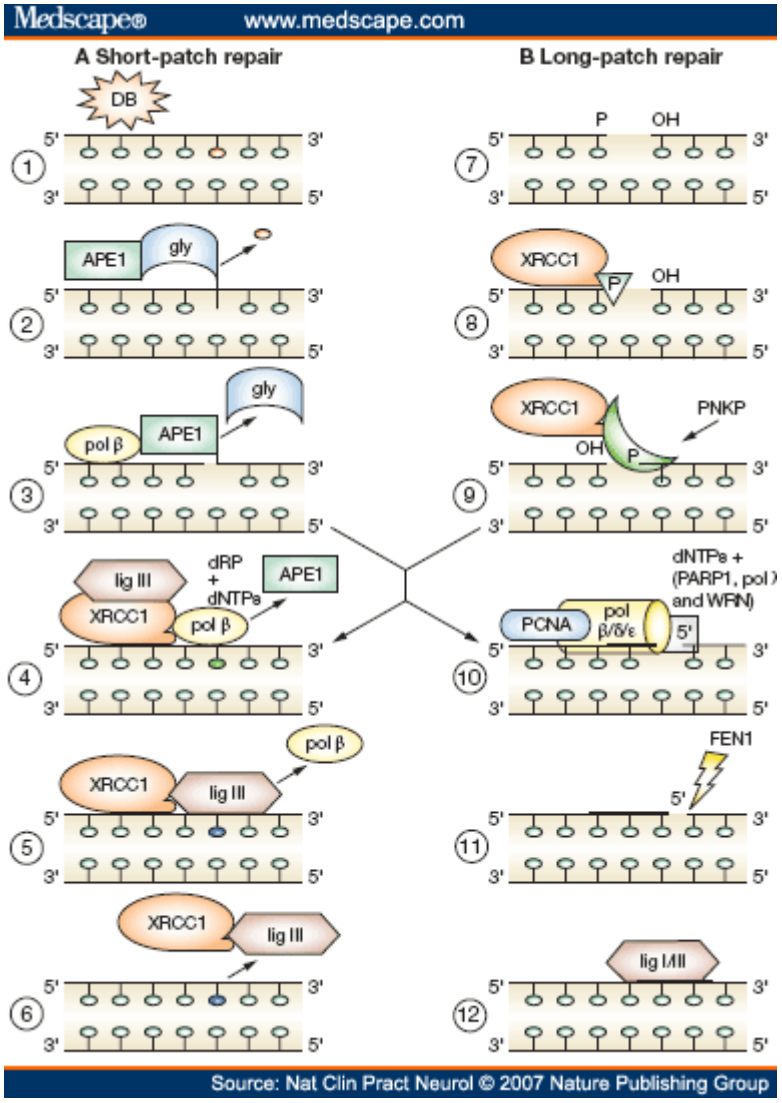

Figure 23: Outline of base excision repair showing the two subpathways: (A) the 'short-patch' or single-nucleotide pathway, and (B) the 'long-patch' pathway (Subba Rao, 2007). There are essentially four steps in the BER pathway. First, when an altered base is detected (1) the surveillance glycosylases remove that base (2). Next, the endonuclease that is specific for abasic site cleaves the strand on the 5' side (3). This is followed by filling in of the gap with a correct nucleotide by DNA polβ, and at the same time releasing the dRP (4). Finally, DNA ligase III ligates the newly introduced nucleotide with the downstream sequence (5), thereby restoring the repaired DNA (6). There are several variations in this process that depend on the nature of the abasic site and the size of the gap to be filled. Sometimes, other DNA polymerases such as DNA polymerase δor ε, along with PCNA, are involved in filling larger sized gaps, also in a strand-displacement manner (long-patch repair [B]; steps 7–12).

It is still not clear how the cell determines which subpathways should be used, but the nature of the base substrates and the glycosylases involves, the types of termini the AP sites, and the specific protein-protein interactions may all be relevant determinants (Dogliotti et al., 2001).

5.2 Nucleotide Excision Repair (NER)

In contrast to the substrate specificity of most DNA glycosylases, NER operates on a large spectrum of base damage. The NER pathway is a multistep process that handles a wide variety of DNA damage, including DNA lesions caused by UV radiation, mutagenic chemicals, or chemotherapeutic drugs that form bulky DNA adducts. Over 30 different proteins are involved in the mammalian NER.

Two NER sub-pathways have been identified: the global genome NER (GG-NER) that detects and removes lesions throughout the genome, and the transcription-coupled NER (TC-NER), which repairs actively transcribed genes. GG-NER and TC-NER involve several common proteins and proceed through the same repair steps, except during recognition of the DNA lesion. In GG-NER this recognition involves the XPC–RAD23B and DDB1–DDB2/XPE proteins, whereas recognition in TC-NER is mediated by cockayne syndrome group A (CSA) (ERCC8) and B (CSB) (ERCC6) proteins after stalling of the RNA polymerase (Hanawalt and Spivak, 2008).

NER begins with the recognition of the DNA lesion, followed by incisions at sites flanking the DNA lesion, and culminates in the removal of the oligonucleotide containing the DNA lesion. Ligation of a newly synthesized oligonucleotide, complementary to the pre-existing strand, serves to fill the gap, thus ending the NER process (Hakem, 2008).

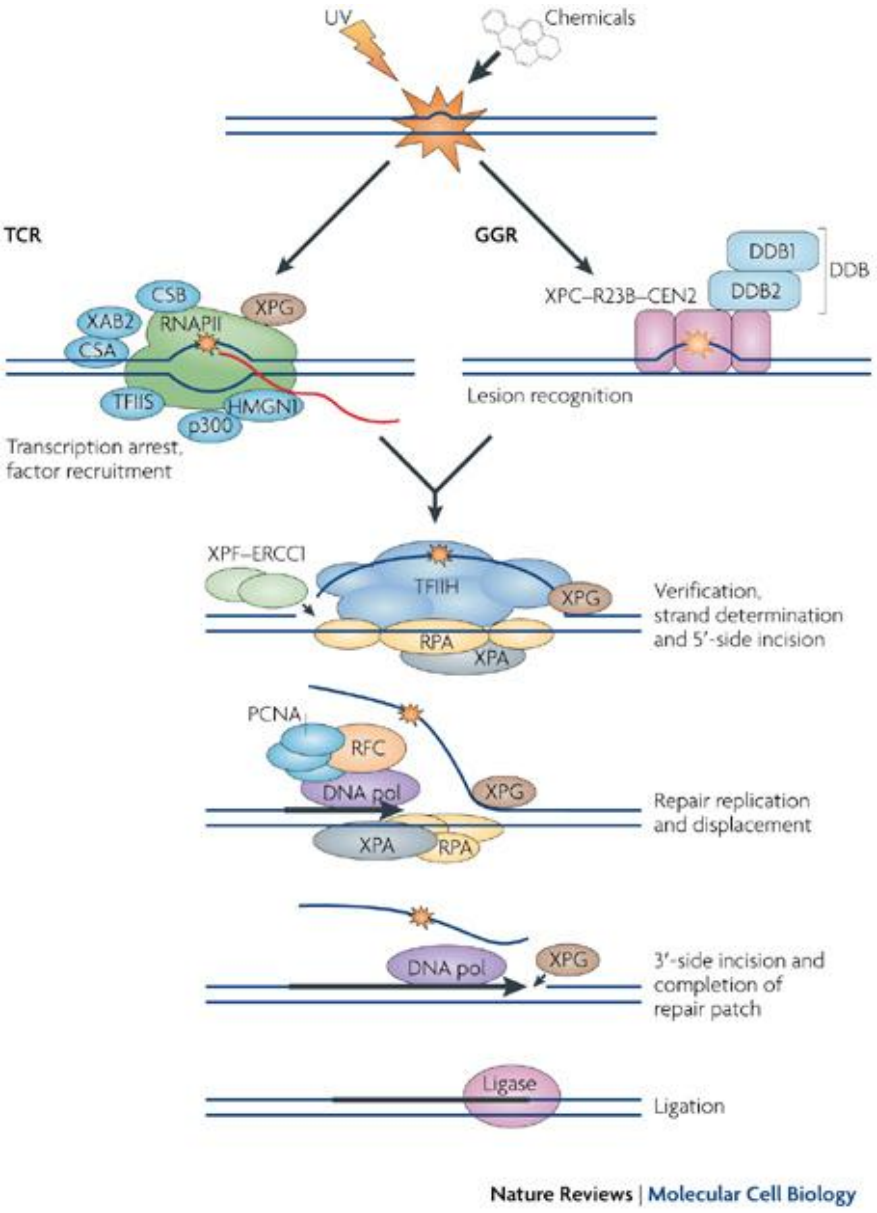

Figure 24: Sequence of events in mammalian NER pathway (Hanawalt and Spivak, 2008). Lesions are initially recognized, either by a translocating RNA polymerase (RNAP) (for transcription coupled repair (TCR); left) or through the binding of the lesion sensor DNA damage-binding-2 (DDB2) — which forms a heterodimer with DDB1 to constitute the DDB complex — and/or XPC in complex with RAD23B and centrin-2 (CEN2) (for global genomic repair (GGR); right). The subpathways converge to the following steps for NER: transcription factor TFIIH is recruited (along with XPG, which stabilizes TFIIH); and the helicase and ATPase activities of its subunits XPD and XPB, respectively, are stimulated for further opening of the damaged DNA. TTDA, another subunit of TFIIH, is required for NER, but its role has not been clarified. Replication protein A (RPA) and XPA might be present before and/or after the appearance of TFIIH, as they have lesion-verification roles and protect the single-stranded DNA in the denatured bubble and stabilize the pre-incision complex. The XPF–ERCC1 endonuclease complex is recruited and incises the damaged DNA strand at the 5′ side of the bubble, whereas XPG incises on the 3′ side. Replication factor C (RFC) loads the processivity factor proliferating cell nuclear antigen (PCNA) to accommodate DNA polymerases (DNA pol) δ, ε and/or κ149 that have been implicated in repair replication.

5.3 Double strand breaks DNA repair

The DNA double-strand break (DSB) is the most critical form of DNA damage and unrepaired or misrepaired DSBs may lead to cell death or changes in the genome stability. There are two primary pathways for the repair of DSBs: Homologous recombination (HR) and non-homologous endjoining (NHEJ). An early step in the repair process is the recognition of DSBs by the binding of repair proteins to the DSB, which protects the ends from extensive degradation and keeps them in proximity to each other, preventing the joining of incorrect ends.

5.3.1 Non-homologous endjoining

NHEJ is the major predominat of DSBs repair because it can function throughout the cell cycle and because it does not require a homologous chromosome (Lieber, 2008). This repair pathway is active especially at the G1, but is error prone. The core protein components of the mammalian NHEJ include the Ku subunits (Ku70 and Ku80), DNA–PKcs, XRCC4, DNA ligase IV (LigIV), Artemis, and the recently identified Cernunnos–XLF (also known as NHEJ1). DNA–PK is composed of the catalytic subunit DNA–PKcs and the heterodimer Ku70/Ku80, important for DNA end binding. DNA–PKc is a serine/threonine kinase that is activated following its recruitment by Ku70/Ku80 to sites of DSBs. Thereafter, Ku can recruit the nuclease, the polymerase (pol μ and pol λ), or ligase (XLF:XRCC4:DNA ligase IV) in any order to work on the 'left' or 'right' end of the DSB. In addition, the nuclease and the ligase activities can work on the top strand somewhat independently of their action on the bottom strand of each of the two DNA ends of the DSB (Ma et al., 2002).

5.3.2 Homologous recombination (HR)

How the cell determines whether HR or NHEJ will be used to repair a break is still an active area of investigation. The HR versus NHEJ determination may be somewhat operational. If a homologue is not present near a DSB during S/G2, then HR cannot proceed, and NHEJ is the only option. During S phase, the sister chromatid is physically very close, thereby providing a homology donor for HR. Outside of S/G2, NHEJ is indeed the markedly preferred option.

In HR the homologous sequence on the sister chromatid is used as a template and HR is therefore a more accurate repair process than NHEJ. HR is initiated by generation of a 3'- single stranded DNA (ssDNA) overhang at the DSB end, after which RAD51 forms nucleoprotein filaments on the ssDNA and mediates homologous pairing of DNA strands and strand exchange reactions between ssDNA and homologous dsDNA (Jackson, 2002).

5.4 DNA mismatch repair (MMR)

DNA mismatch repair (MMR) is a highly conserved DNA repair system that greatly contributes to maintain genome stability through the correction of mismatched base pairs. The major source of mismatched base pairs is replication error, although it can arise also from other biological processes (Modrich and Lahue, 1996). MMR is a highly conserved biological pathway with strong similarities between human MMR and prototypical *E. coli* MMR (Kunkel and Erie, 2005). These similarities include substrate specificity, bidirectionality, and nick-directed strand specificity. Several human MMR proteins have been identified based on their homology to *E. coli* MMR proteins. These include human homologs of MutS, MutL, EXO1, single-strand DNA-binding protein RPA, proliferating cellular nuclear antigen (PCNA), DNA polymerase δ (pol δ), and DNA ligase I (Li, 2008).

5.5 DNA direct repair (DDR)

Single-step reversal of DNA damage is performed by proteins that are able to recognize DNA lesions and catalytically restore DNA to its original state. The three families of proteins that are known to directly reverse DNA damage include the enzyme families photolyases and oxidative demethylases (dioxygenases) as well as the nonenzymatic O^6-mG DNA methyltransferases. The enzymatic direct reversal of damage by photolyases and dioxygenases represents the most cost-efficient and least error-prone DNA repair mechanisms known. On the other hand, the DNA methyltransferases are involved in extremely costly repair, where the alkyl group on DNA is irreversibly transferred to the scavenger protein, which then becomes nonfunctional and tagged for degradation (Dalhus et al., 2009).

Aim of the study

Epidemiological studies have demonstrated an inverse relationship between Se intake and cancer incidence and/or mortality. However, the molecular mechanism underlying the cancer chemopreventive activity of Se compounds remain largely unknown. The objective of this study was to investigate the effect of selenocompounds at low doses on DNA repair capacity in the p53-proficient LNCaP prostate cancer cells.

The first part of the work was devoted to study the effect of two selenocompounds, sodium selenite (SS) and selenomethionine (SM) on the cytotoxic and genotoxic properties of various oxidative and non oxidative stresses. Several stress-inducers (UVA, UVC, H_2O_2 and MMS) were selected fot their ability to induce different classes of DNA lesions. DNA damage was investigated by two complementary approaches: the Comet assay and LC-MS/MS analysis. This step aimed at determining which kind of cellular stress will be modulated by selenium supplementation.

The second part of our investigation was devoted to the influence of selenium supplementation on DNA repair capacity. Therefore, we measured the repair rate of several DNA damaging agents which are handled by different repair pathways. DNA repair capacity was investigated by two *in vitro* approaches: the modified Comet assay to measure the excision activity of the protein extracts and the multiplexed ODN assay which allows the quantification of the excision capacity of several specific base damages. The purpose was to understand which DNA repair system was modulated by selenium supplementation.

Finally, the third part of our work was devoted to the optimization of Host Cell Reactivation assay (HCR) to study the DNA repair capacity *in cellulo*, in order to

target the partners involved in the signalling pathway affected by selenium supplementation.

This whole study gave us a more mechanistic vision of the influence of selenium pre-treatment on DNA damage and DNA repair. Moreover, we better understand which DNA repair system is affected by selenium in the cellular model adopted. Furthermore, Se-treated cells exhibited increased oxidative DNA repair activity, indicating a novel mechanism of selenium action. Our work provides an extended view of the protection afforded by selenium against cancer: selenium not only behaves as an antioxidant but also enhances of DNA repair capacity.

Chapter 2 – Evaluation of the protective effect of selenium supplementation on different DNA damaging agents in the LNCaP cells

The most robust results of the NPC Trial came from the analyses of the complete data for prostate cancer incidence. It showed that dietary supplementation with 200 μg/day of yeast-enriched selenium resulted in a 63% reduction in prostate cancer risk (Duffield-Lillico et al., 2003). Moreover, because dogs and humans are the only two species in which prostate cancer occurs spontaneously with appreciable frequency, Waters et al. examined the effects of dietary selenium supplementation on DNA damage in elderly beagle dogs and found efficient protection (Waters et al., 2003). Se-mediated protection of the prostate against cancer has been well-documented (Chan et al., 2009).

For these reason, we focus our work on cultured prostate cells. LNCaP, PC-3 and DU-145 are commonly used prostate cancer cell lines. LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma. Moreover, the proliferation of LNCaP cells is androgen-dependent but the proliferation of PC-3 and DU-145 cells is androgen-insensitive. Unlike the other two cell lines LNCaP are wild-type for gene suppressor p53. Therefore, in the present work LNCaP cells were used as an *in vitro* tool for assessing the protection afforded by Se against genotoxicity of several stress agents.

We used two different selenocompounds, sodium selenite (SS) an inorganic Se-derived and selenomethionine (SM) an organic one. These compounds are the two most common selenocompounds used in several epidemiological studies and present a different metabolism in cells.

Selenium supplementation could increase the levels of several antioxidant selenoproteins. Therefore, we analysed the expression and the activity of two best characterized selenoenzymes, glutathione peroxidase (GPx1) and thioredoxin reductase (TrxR).

Before assessing DNA damage caused by exposure to several stresses, it was necessary to determine the optimal selenocompounds concentration to cell treatment and the

cytotoxicity of the different stress agents. These stress inducers were chosen in order to test a large variety of types of DNA damage handled by different repair systems.

These results showed that low doses of Se pre-treatment stimulate selenoprotein synthesis, protect against toxicity and oxidative DNA damage induced by UVA or H_2O_2 but not by MMS or UVC.

1. Determination of optimal selenium compounds concentration in LNCaP cells

The optimal concentration is the value for which we have maximum protection at the lowest concentration. The optimal working concentration of SS and SM will be used in all following experiments. The cells were plated for three days and then irradiated with a growing dose of UVA (Figure 25). UVA radiation was chosen because it is known to induce at least two different DNA lesions (8-oxoGua and CPDs). In addition, UVA is an efficient inducer of oxidative stress.

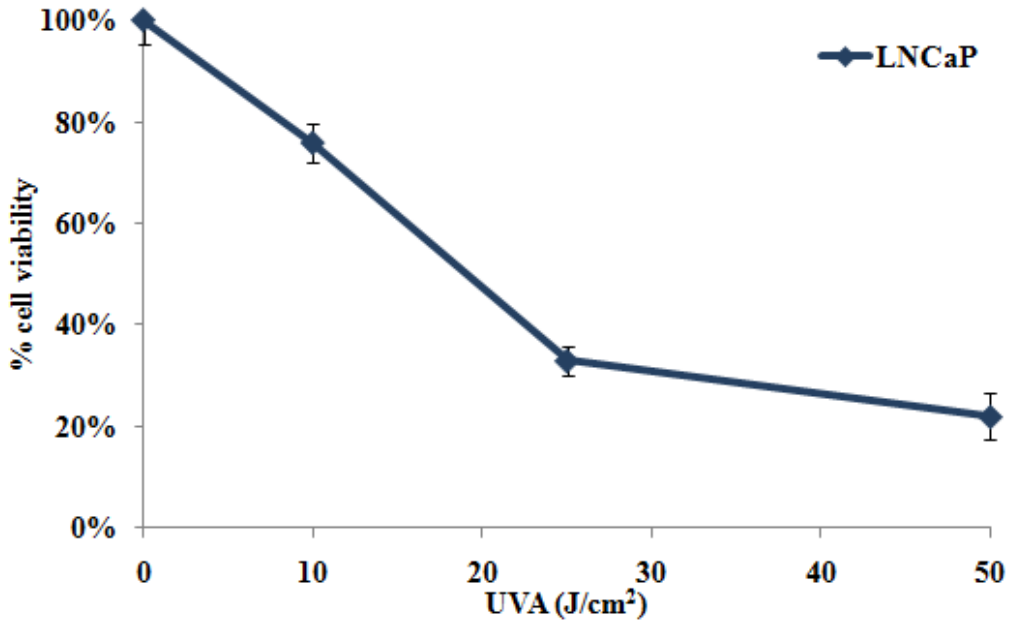

Figure 25: Cell survival response to increasing dose of UVA radiation in LNCaP cells. Cell viability was determined by the MTT assay and data were presented relative to the sham-irradiated control cells. The MTT test was performed 24 h after irradiation.

For the determination of the optimal concentrations of two selenocompounds we used UVA dose of 50 J/cm² that correspond to a 20% cell survival.

The cells were pre-treated three days with different concentration of SS and SM, and then irradiated with UVA at 50 J/cm². The pre-treatment time was chosen in order to avoid influence of selenium metabolism on cell cycle (Zeng, 2009). Twenty-four hours after the irradiation, the cell viability was measured by MTT assay (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide). This colorimetric assay is based on the measure of the enzyme mitochondrial activities which reduce MTT to purple formazan in living cells. The absorbance of non-irradiated control sample determined the 100 % of living cells and the absorbance of irradiated samples was reported to this value. This survival assay reflects at the same time the necrosis and the apoptosis mechanisms.

Figure 26 shows the protection afforded by a growing concentration of SS after UVA irradiation. A protective effect was observed at 10 nM and a plateau of protection is obtained from 30 nM. So for the following experiments, we used a dose of 30 nM of SS. The same figure also shows the absence of toxicity of SS over the tested range of concentrations.

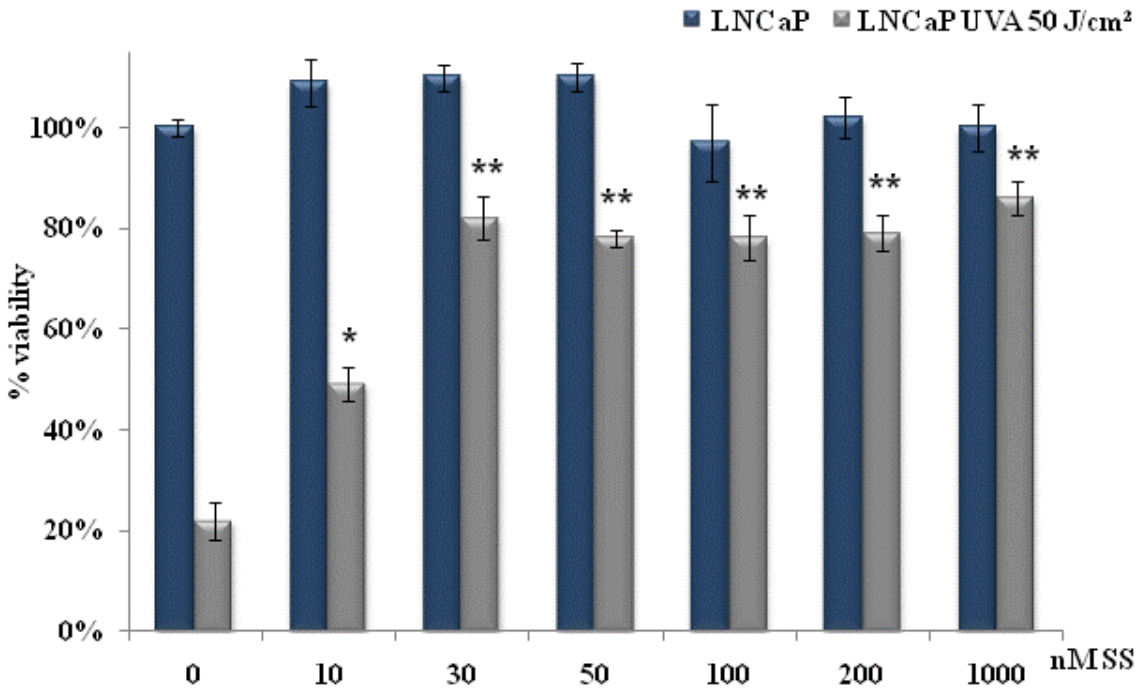

Figure 26: Cell survival response to increasing concentrations of SS after UVA radiation in LNCaP cells; * p < 0.05, ** p < 0.01 compared to LNCaP without SS. Cell viability was determined by the MTT assay and data were presented relative to sham-irradiated control cells. LNCaP cells were pre-treated

with growing concentrations of SS for 72 h and then were irradiated with UVA at 50 J/cm². The MTT was performed 24 h after irradiation.

Figure 27 shows the protective effect of SM after UVA irradiation. Unlike SS, the optimal SM concentration was found to be 10 µM. The optimal concentrations of SS and SM were really different from each other, SM concentration was 300 fold higher than SS. This could be explained by the different absorption and metabolism of these two different compounds. A sligth toxicity of SM can be observed at 20 and 50 µM.

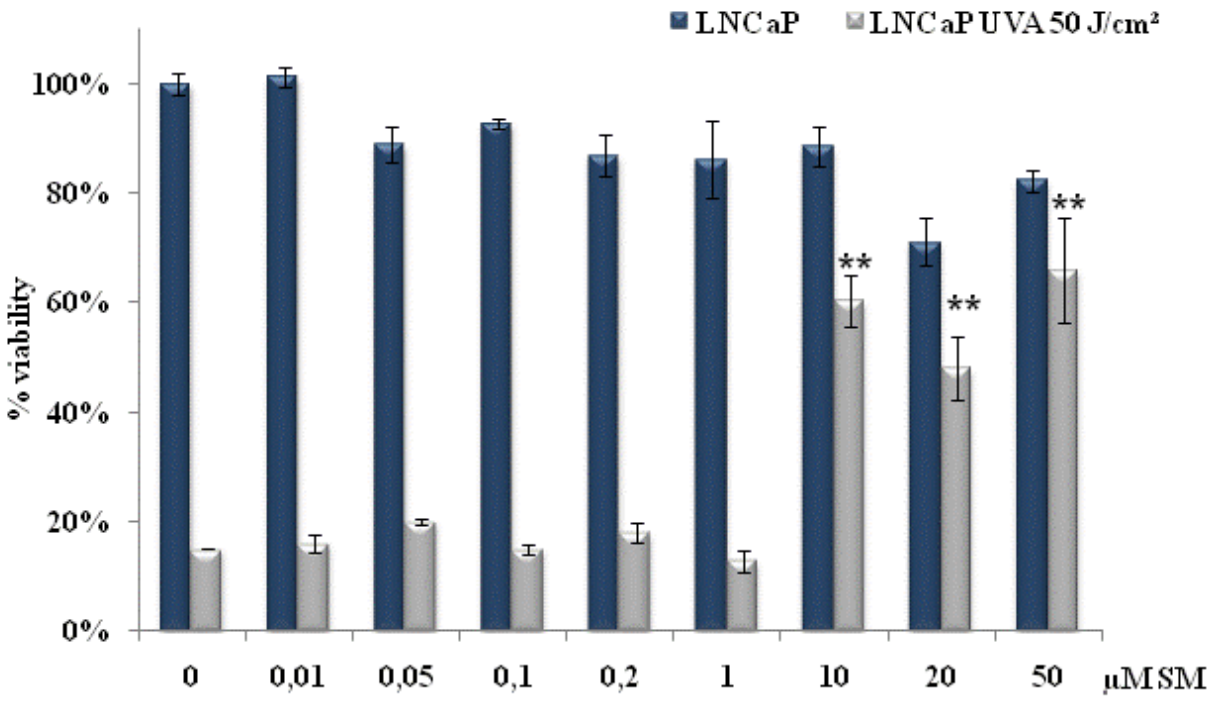

Figure 27: Cell survival response to increasing concentrations of SM after UVA radiation in LNCaP cells; ** p < 0.01 compared to LNCaP without SM. Cell viability was determined by the MTT assay and data were presented relative to sham-irradiated control cells. LNCaP cells were pre-treated with different concentrations of SM for 72 h and then were irradiated with UVA at 50 J/cm². The MTT was performed 24 h after irradiation.

In summary, cytoprotective effect was observed for both SS and SM upon UVA irradiation.

2. The effect of Se supplementation on selenoenzymes

Cytosolic GPx (GPx1) is a selenium-containing antioxidant enzyme composed of four identical subunits, and each subunit contains one selenocysteine residue. Mammalian TrxR is an homodimer of selenoproteins.

The thioredoxin and glutathione systems are involved in a variety of redox-dependent pathways such as providing reducing equivalents for ribonucleotide reductase (the first step in DNA biosynthesis) and peptide methionine sulfoxide reductase; antioxidant defense and regulation of the cellular redox state. In addition, these two systems regulate activities of various transcription factors, kinases, and phosphatases, and they were implicated in the redox control of cell growth and death, transcription, cell signaling, and other processes.

In order to evaluate if LNCaP cells were sensitive to selenium pre-treatment, we carried out several assays to measure the modulation of GPx1 and TrxR, from the gene expression to protein activities.

2.1 Gene expression of GPx1 by qPCR

The expression levels of GPx1 enzyme was measured using real-time quantitative PCR, which allows for the relative quantification of a target gene under SS and SM condition compared to NT condition by normalizing to one housekeeping gene (S18) that is considered stably expressed in all conditions. Figure 28 shows the gene expression of GPx1 for SS and SM pre-treatmentcompared to NT. GPx1 mRNA was significantly over-expressed (~3 fold) in cells pre-treated with SS. Whereas, cells pre-treated with SM did not show an increase expression compared to NT. Despite SM concentration is ~300 fold higher than SS, we can observe an increase of GPx1 expression 2 fold higher for SS pre-treatment than SM.

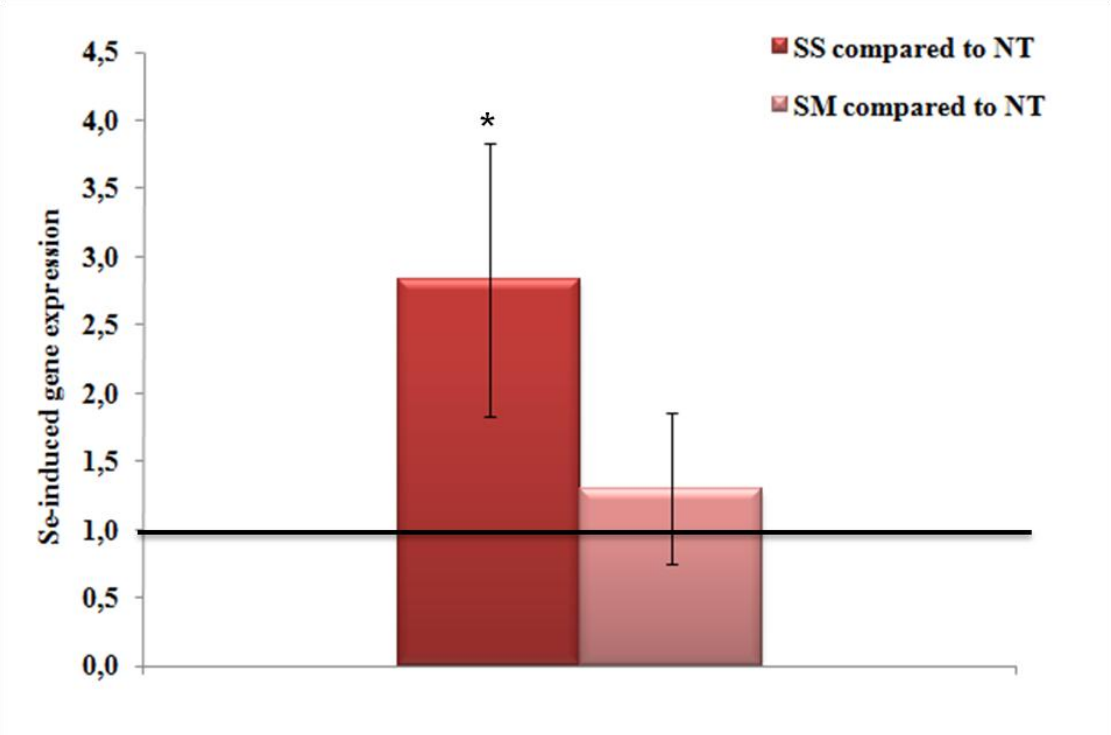

Figure 28: Absolute GPx1 expression in LNCaP cells pre-treated with SS or SM. LNCaP cell lines were cultured for 72 h with or without SS or SM. Total RNA was extracted and subsequently reverse-transcribed. A total of 20 ng of corresponding cDNA were used to detect specific gene expression using real-time qPCR. Absolute gene expression in SS or SM conditions compared to NT cells was calculated using REST. The mean of the corresponding expression ratios was calculated, and a Student's t-test was performed. * $p<0.05$ compared to NT

2.2 Protein expression analysis by Western Blotting

In order to determine whether modulation in gene expression was reflected at the protein level, Western blot experiments were performed to compare the level of GPx1 and TrxR expression between Se-pretreated cells and untreated ones.

The Western blot is a technique used to identify a protein of interest from protein extracts. It is based on the ability of the protein to bind to specific antibodies.

Cells were pre-treated or not with the two selenocompounds for 72 h and then were collected; total proteins were extracted.

Figure 29 represents a picture of the membrane and we can observe that the GPx1 band is more intense in SS- and SM-treated cells compared to NT. Moreover, the TrxR band is a little bit more intense for SS and SM compared to NT.

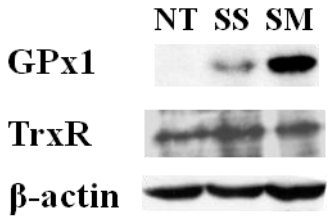

Figure 29: Representative image of Western blot analysis performed on total extracts from NT, SS and SM conditions. Equal amount of total protein were western blotted by using GPx1 and TrxR and β-actin antibodies. The relative abundance of each targeted protein is displayed in photo with their corresponding same membrane-revealed β-actin controls.

To quantify the bands, we used a software (ImageJ) and then we normalized protein expression with respect to the β-actin (it is equally expressed in all conditions) for each condition.

Figure 30 represents the ratio between the protein value for SS or SM conditions and NT one. The results showed that SS and SM pre-treatment induce an increase of ~9 and ~18 fold of GPx1 protein contained in LNCaP cells. Unlike qPCR results, GPx1 protein expression is ~2 fold highe for SM pre-treated cell than SS.

TrxR protein expression is only slightly increased after selenium pre-treatment compared to NT. Moreover, we did not observe a significantly difference in TrxR expression between two selenocompounds treatments.

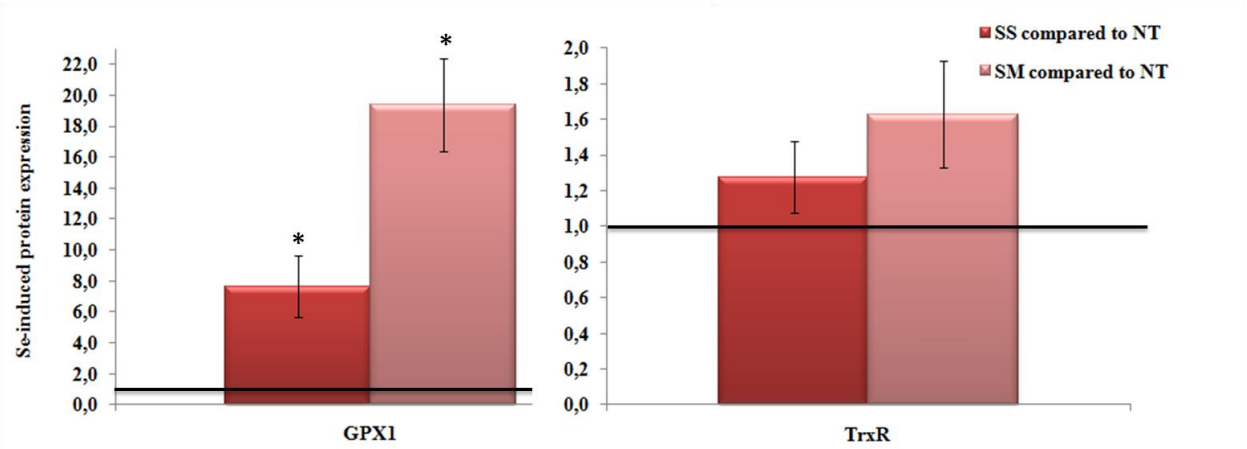

Figure 30: Protein expression levels of GPx1 and TrxR Band quantification allowed the comparison between SS or SM to NT, and revealed that GPx1 was significantly over-expressed in SS and SM cells, while the expression of TrxR appeared slightly enhanced.

2.3 Activities assay

Last, we investigated activities of GPx and TrxR, by colorimetric and spectroscopical assays. The Thioredoxin Reductase Assay Kit uses a colorimetric assay for the determination of thioredoxin reductase activity. It is based on the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm. This Glutathione peroxidase kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions.

The modulation of the activity of the GPx1 and TrxR selenoenzymes by low concentrations of SS and SM in LNCaP cells are summarized in Table 3. Se supplementation of LNCaP cells with either SS or SM significantly increased the activities of TrxR (~3-fold) and GPx1 (≥ 2-fold). TrxR activity is the same for either SS and SM, but GPx1 activity is slightly more increased in LNCaP cells pre-treated with SM than SS.

	NT	**SS**	**SM**
TrxR (U/mg protein)	**0.025±0.005**	**0.081±0.009***	**0.078±0.002***
GPx1(U/mg protein)	**9.0±2.1**	**18.7±5.6***	**25.7±2.4***

Table 3: GPx and TrxR activities in LNCaP cells and effects of Se supplementation.* $p<0.05$ vs NT.

3. Effect of low-doses of selenium supplementation on cell survival

The cytotoxic effect of different DNA damaging agents in the LNCaP cells pre-treated or not with SS or SM was evaluated using short and long term assays.

The short term assay was carried out using the MTT assay which evaluated the cell survival 24 h after stress.

On the other hand, the clonogenic cell survival allowed us to study the effectiveness of specific agents on the survival and proliferation of cells 12 days after stress.

The several stress-inducers were chosen for their ability to induce very different classes of DNA damage, such as oxidized and alkylated lesions; bulky adducts and double strand breaks.

3.1 Cytotoxicity assay

For all cytotoxicity assays, cells were pre-treated with SS or SM during 72 hours.

After 3 days cells were treated with several DNA-damaging agents and 24 h after that we measured cell viability.

Moreover, to understand whether selenium supplementation was fundamental to prevent cellular death or it was able to ameliorate cell survival also after treatment we also carried out selenium post-treatment. That is, LNCaP cells were plated for 72 hours, then cells were stressed and immediately after stress post-treated or not with SS and SM for 24h.

Three biological replicates were tested in three independent experiments.

3.1.1 Oxidative DNA damaging agents

Figure 31 shows the viability of the cells irradiated with increasing doses of UVA. We observed that cells pre-treated with selenium exhibited much better survival than controls after UVA irradiation. For example, after exposure to 25 J/cm² of UVA, SS and SM pre-treated cells were fully viable whereas viability of NT cells was only 30%. In contrast, post-treatment with selenocompounds had no effect on cell survival.

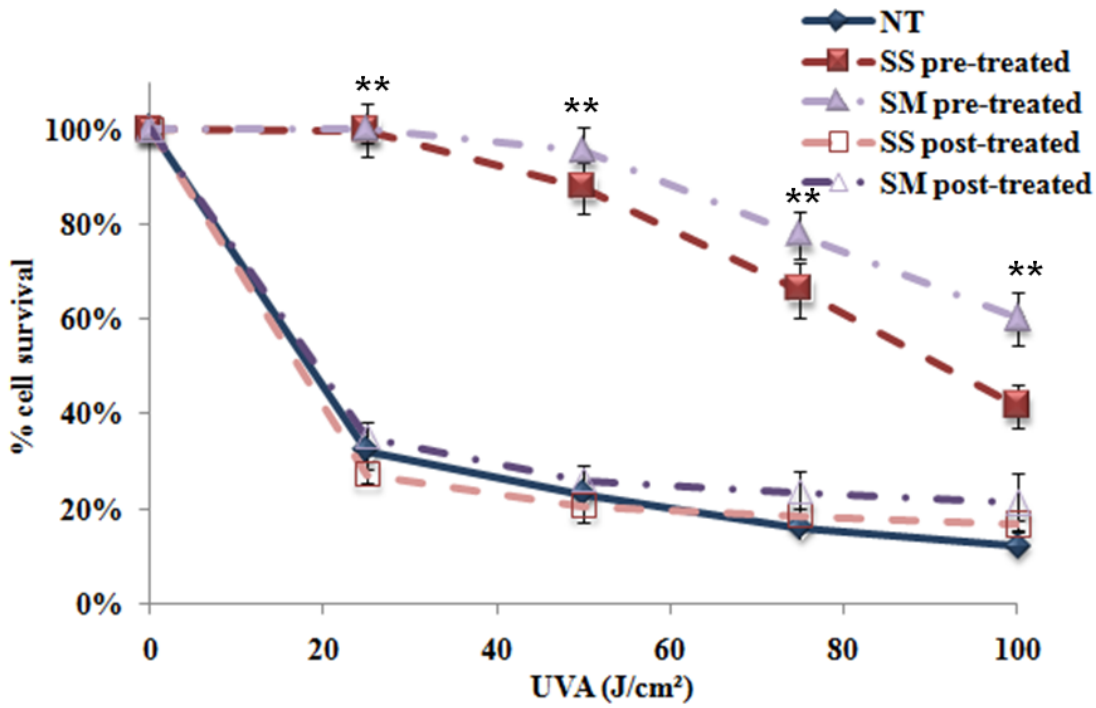

Figure 31: Cytotoxicity of several doses of UVA in LNCaP cells pre-treated or post-treated with SS or SM. **p<0.01 vs NT. Cell viability was determined by the MTT assay and data were presented relative to sham-irradiated control cells. LNCaP cells were pre-treated and post-treated with 30 nM SS or 10 µM SM for 72 h and 24 h. Cells were irradiated with UVA.

Similar observations were made with H_2O_2 (Figure 32). The H_2O_2 incubation was carried out on ice for 30 min. Pre-treatment with SS and SM resulted in greater cell survival when compared to NT. The IC_{50} of H_2O_2 in NT cells was 200 µM whereas SS and SM pre-treated cells had an IC_{50} of 5 mM of H_2O_2.

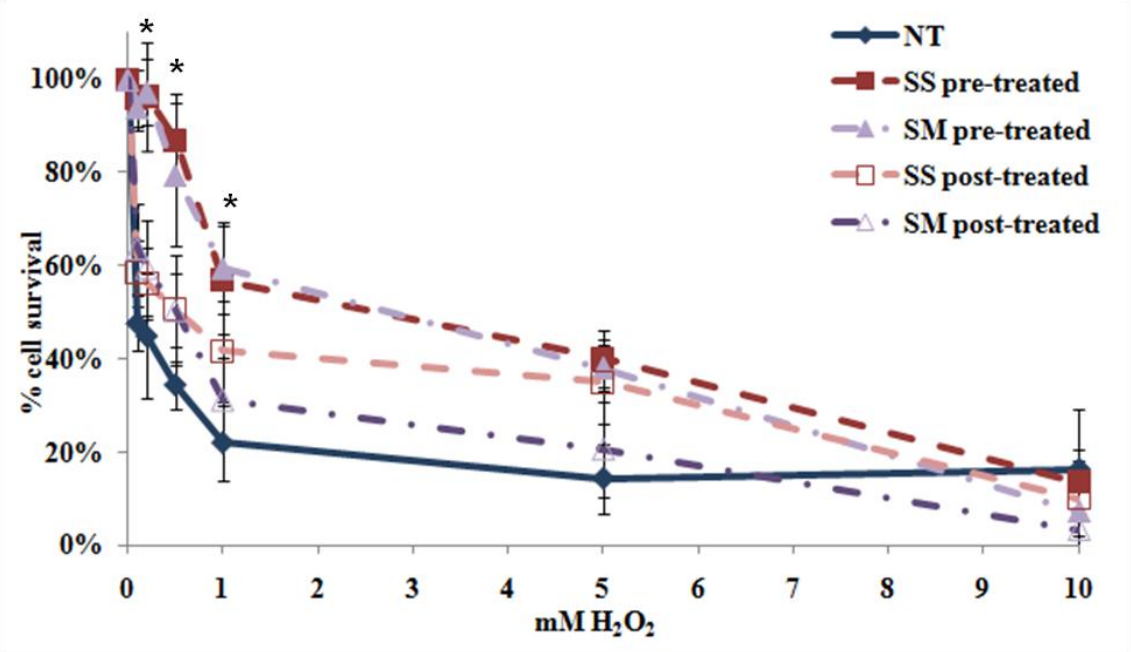

Figure 32: Cytotoxicity of various concentrations of H_2O_2 in LNCaP cells pre-treated or post-treated with SS or SM. *p< 0.05 **p<0.01 vs NT. Cell viability was determined by the MTT assay and data were presented relative to control unexposed cells. LNCaP cells were pre-treated and post-treated with 30 nM SS or 10 µM SM for 72 h and 24 h. Cells were incubated with H_2O_2 (30 min on ice).

To confirm the protection of selenium compounds against oxidative stress, we incubated cells with different concentration of potassium bromate ($KBrO_3$) for 24 h at 37°C. This compound induces oxidative stress and ROS generation. $KBrO_3$, as shown in Figure 33, was less cytotoxic for NT than H_2O_2 at the same concentration. The IC_{50} of NT was 2 mM while it was 4 mM for both SS and SM. At higher concentration of $KBrO_3$ we observed a significantly better cell survival with SS and SM pre-treatment.

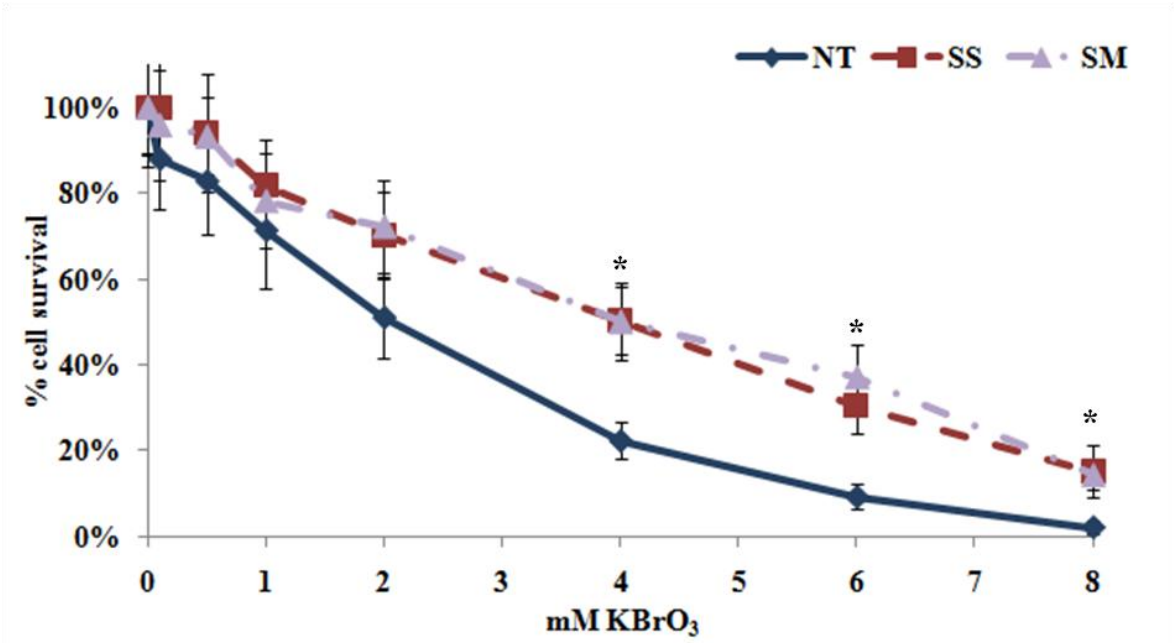

Figure 33: Cytotoxicity of increasing concentrations of $KBrO_3$ in LNCaP. *p< 0.05 vs NT. Cell viability was determined by the MTT assay and data were presented relative to control unexposed cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were incubated with $KBrO_3$ (24 h at 37°C).

3.1.2 Alkylating DNA damaging agents

The next type of stress was a monofunctional alkylating agent, namely MMS. Compared to the previous treatments, the MMS incubation did not highlight any effects of SS and SM treatment on cell survival (Figure 34).

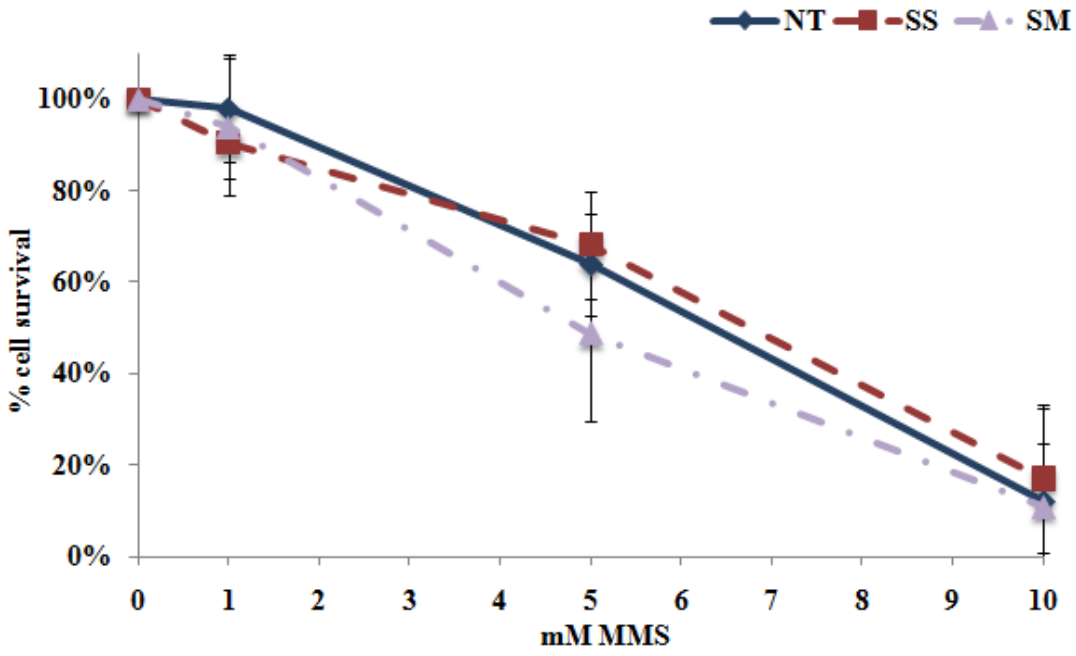

Figure 34: Cytotoxicity of growing concentrations of MMS in LNCaP cells pre-treated with SS or SM. Cell viability was determined by the MTT assay and data were presented relative to control unexposed cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were incubated with MMS (15 min at 37°C).

3.1.3 Bulky DNA damaging agents

In order to complete UVA data, we investigated the effect of UVB and UVC on LNCaP cells with or without selenium pre-treatment. In contrast to UVA, there was not protective effect of SS and SM against cell death after exposure to UVB and UVC (Figure 35). The UVB and UVC radiation cause only limited ROS formation and oxidative stress to cells. On the other hand, the main lesions induced by UVB and UVC are bulky adducts (CPDs and (6-4)PP).

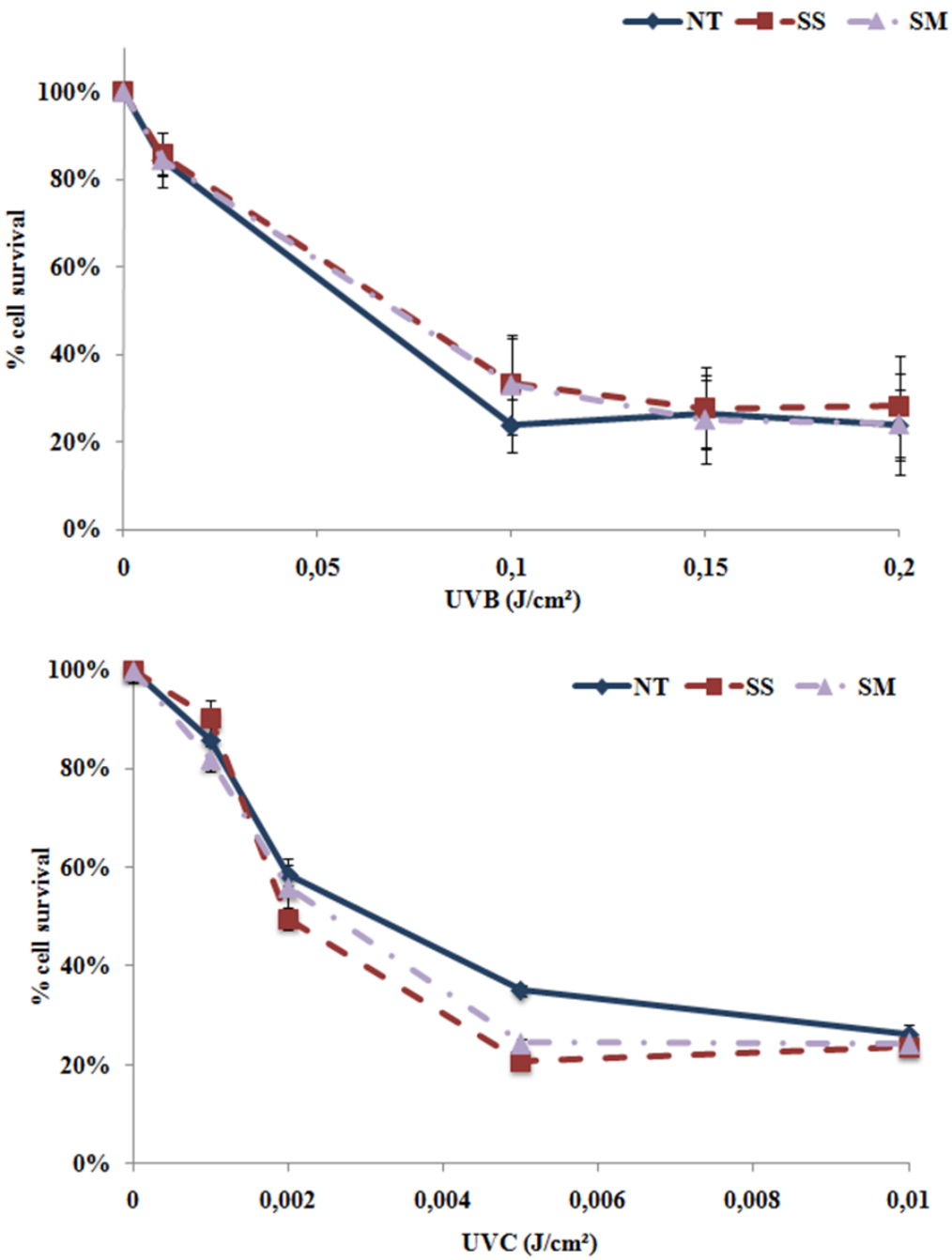

Figure 35: Cytotoxicity of increasing doses of UVB and UVC in LNCaP cells pre-treated with SS or SM. Cell viability was determined by the MTT assay and data were presented relative to sham-irradiated control cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were irradiated with UVC and UVB.

Another kind of agent inducing bulky lesions is cis-platin that is a chemotherapeutic drug. LNCaP cells were incubated with increasing concentrations of cis-platin for 24 h at 37°C. Like UVB and UVC results, selenium pre-treatment did not affect cell survival

after cis-platin incubation (Figure 36). The DNA lesions induced by this compound are voluminous and could be form inter- or intrastrand crosslink that principally deformed the double helix.

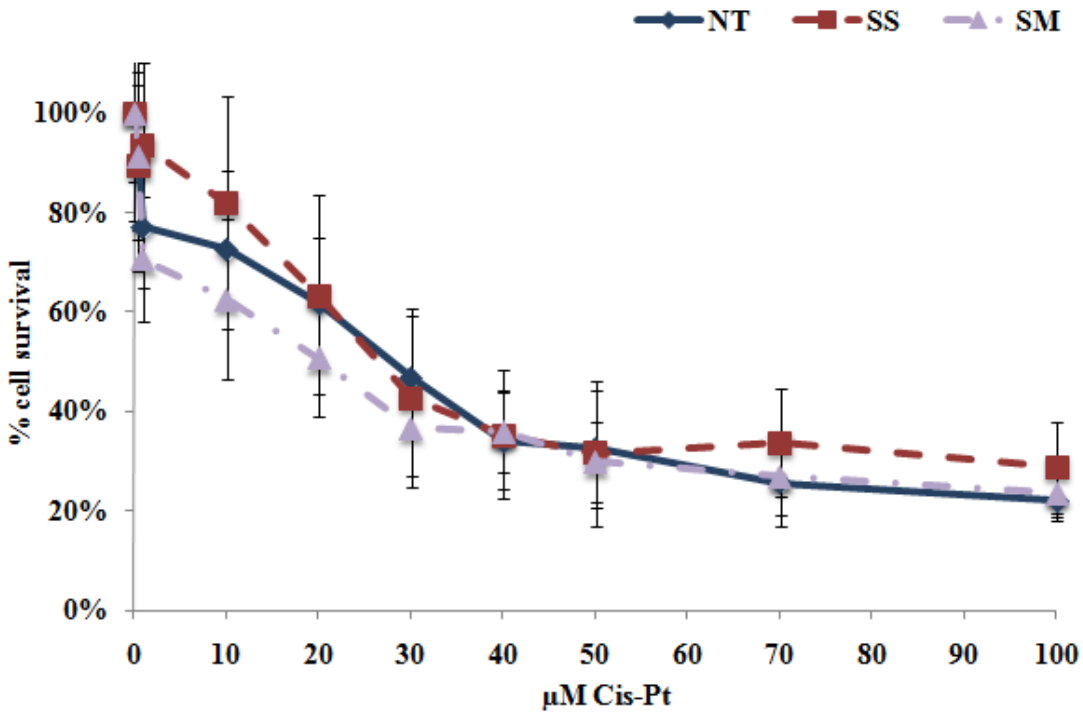

Figure 36: Cytotoxicity of increasing concentrations of Cis-Pt in LNCaP cells pre-treated with SS or SM. Cell viability was determined by the MTT assay and data were presented relative to control unexposed cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were incubated with Cis-Pt.

3.1.4 Double strand breaks DNA damaging agents

Double-strand breaks, in which both strands in the double helix are cleaved, are particularly hazardous to the cell because they can lead to genome rearrangements and lethality. Among several agents that cause DBS we choose to use γ-rays and the glycopeptide antibiotic bleomycin.

The cells irradiated with γ-ray, using a source of ^{60}Co, did not show a decrease in short-term cell survival with increasing doses and also there is no effect of selenium treatment, as shown in Figure 37. It should be emphasized that 10 Gy is often lethal to

most cell types. Such results are based on clonogenic survival cell assay that is more sensitive than MTT. Moreover, LNCaP cell are reported to be radiorsistant.

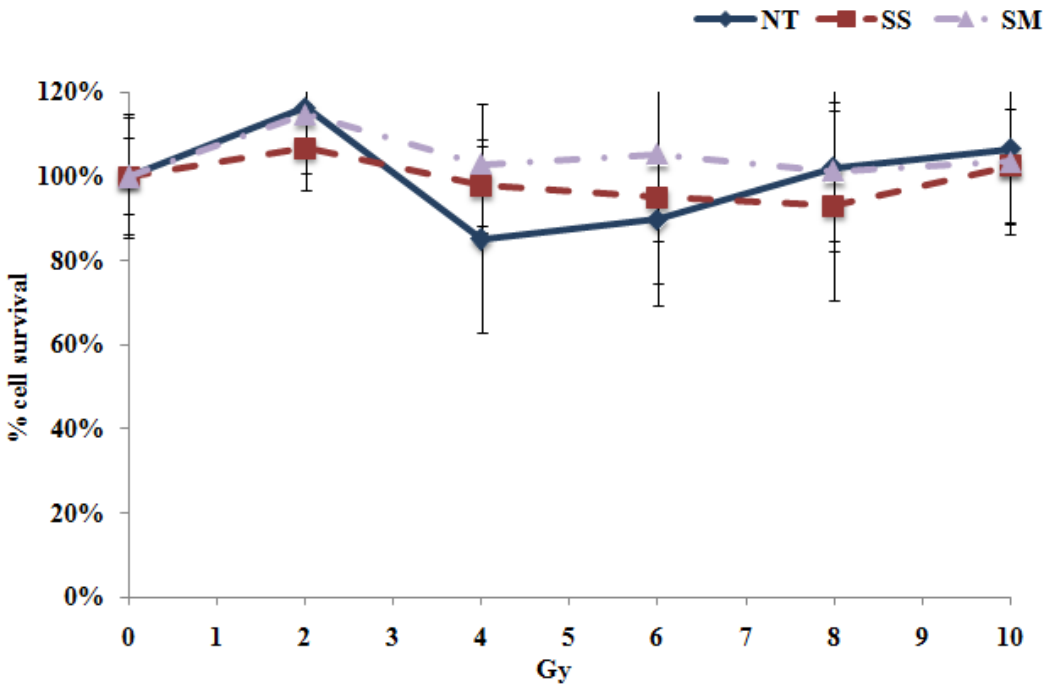

Figure 37: Cell survival after exposure to γ-ray doses in LNCaP cells pre-treated with SS or SM. Cell viability was determined by the MTT assay and data were presented relative to sham-irradiated control cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were irradiated with a source of ^{60}Co.

Bleomycin induces double and single DNA strand breaks through an oxygen-radical-dependent mechanism. Figure 38 show that SS and SM did not influence cell survival. Bleomycin also generates ROS but in contrast to other oxidized agent its mechanism of action takes place within the double-helix.

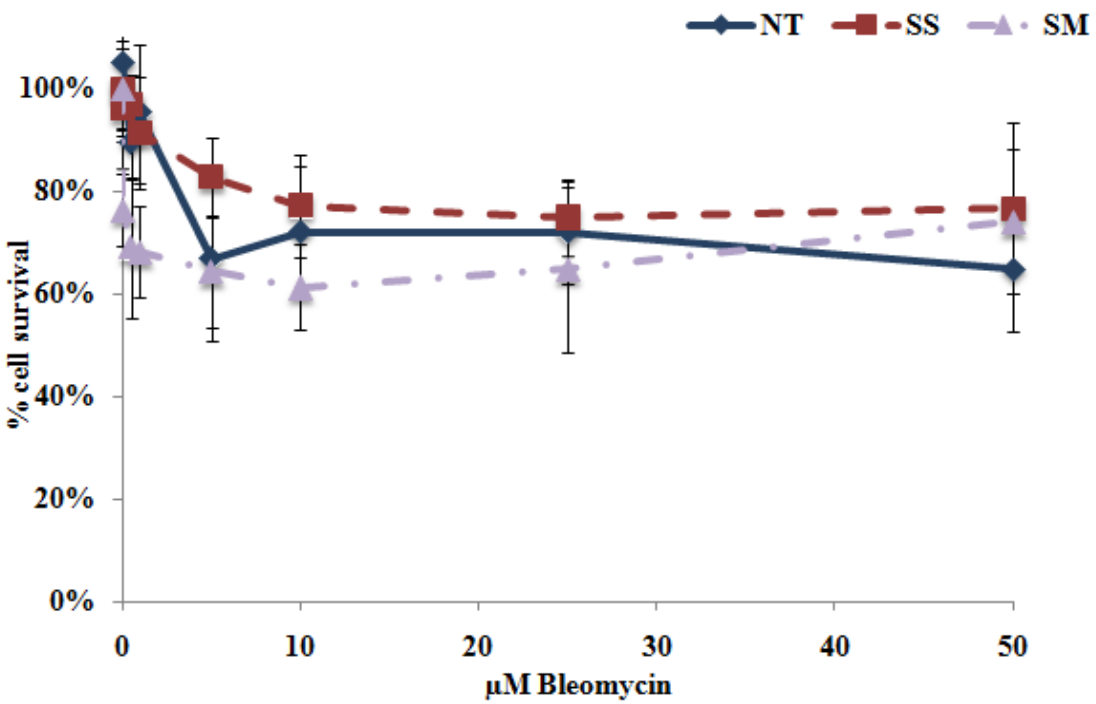

Figure 38: Cytotoxicity of growing concentrations of Bleomycin in LNCaP cells pre-treated with SS or SM. Cell viability was determined by the MTT assay and data were presented relative to control unexposed cells. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were incubated with bleomycin (24 h at 37°C).

3.2 Clonogenic cell survival assay

The next step was to confirm the Se-mediated modulation of the cellular response to stress by a clonogenic assay. This cell survival assay is based on the ability of a single cell to grow into a colony, and includes long term effects of exposure (12 days).

The clonogenic assay was carried following treatment with UVA, H_2O_2 or $KBrO_3$ for which selenium protection was shown in short-term survival. The results of all three treatments showed a significantly Se-protection of cellular response to oxidative stresses (Figure 39).

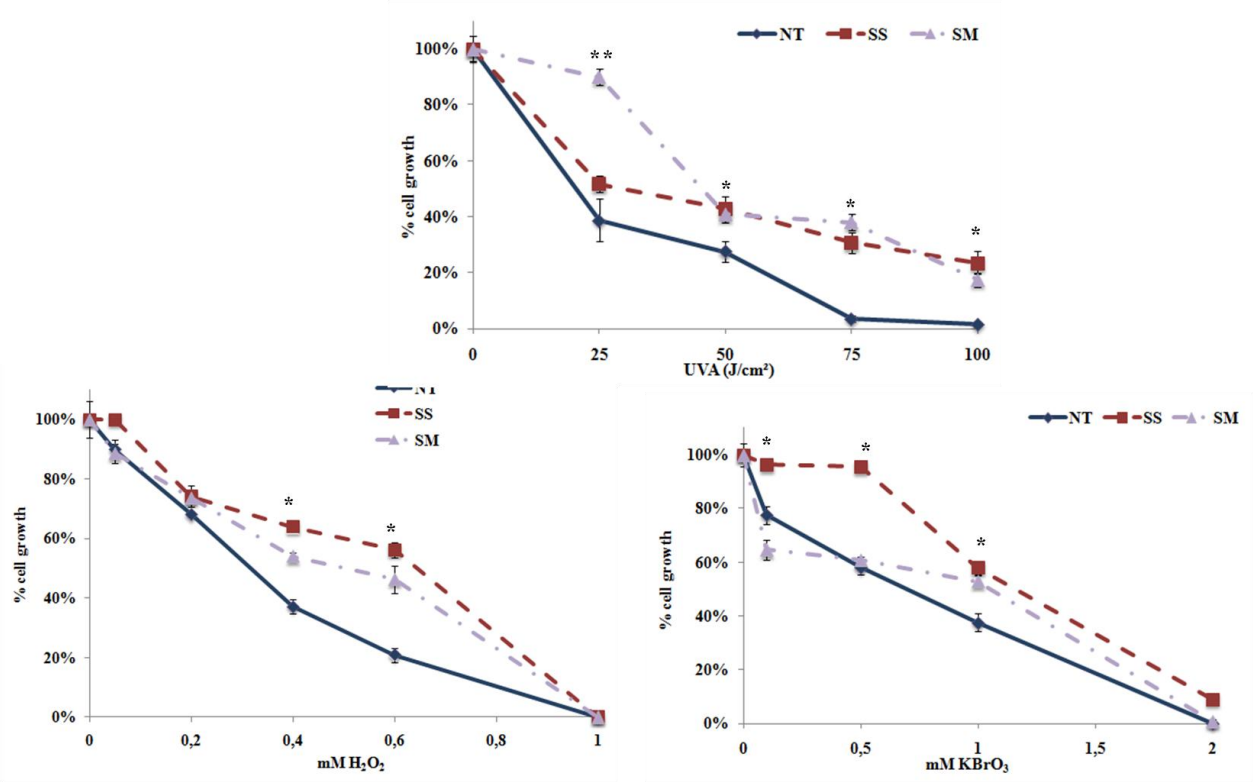

Figure 39: Clonogenic assay in LNCaP cells pre-treated or not with SS or SM. * p < 0.05 and ** p< 0.01 vs NT. Cells were pre-treated with SS or SM for 72 h, then irradiated with growing dose of UVA, or incubated with H_2O_2 (30 min on ice) or $KBrO_3$ (24 h at 37°C) and re-plated in 100 mm petri dishes with 200 cells for 12 days.

Figure 40 shows an example of the colonies formation after UVA irradiation for all three conditions. It is clearly visible that a higher number of colonies are present for SS and SM conditions compared to NT.

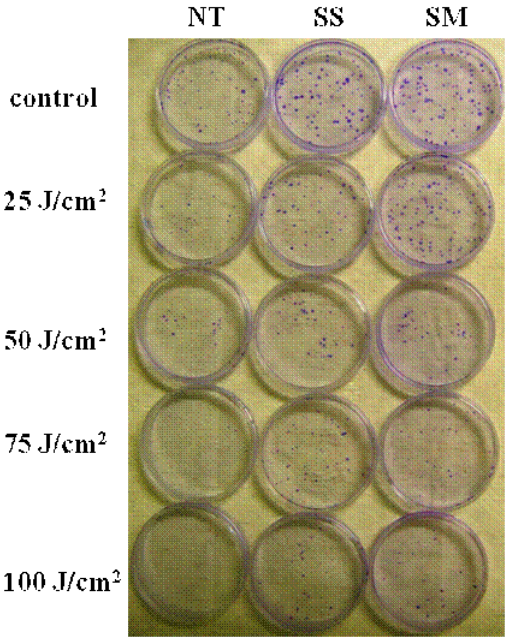

Figure 40: Representative picture of clonogenic cell survival assay after UVA irradiation in LNCaP cells.

The same experiment was carried out after UVC, UVB and MMS treatment. We did not observe any effect of Se-treatment on cell growth after growing dose of UVC (Figure 41).

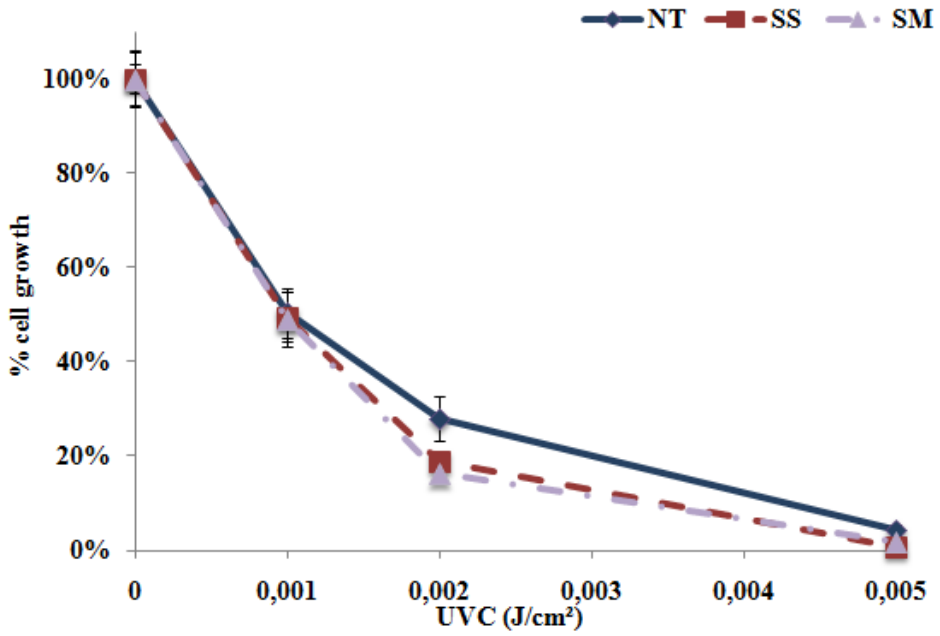

Figure 41: Clonogenic assay in LNCaP cells after UVC irradiation. Cells were pre-treated with SS or SM for 72 h, then irradiated with growing UVC doses and re-plated at a density of 200 cellules for 12 days.

The same results were obtained after MMS and UVB treatment. We observe, in Figure 42, the same response for all three conditions.

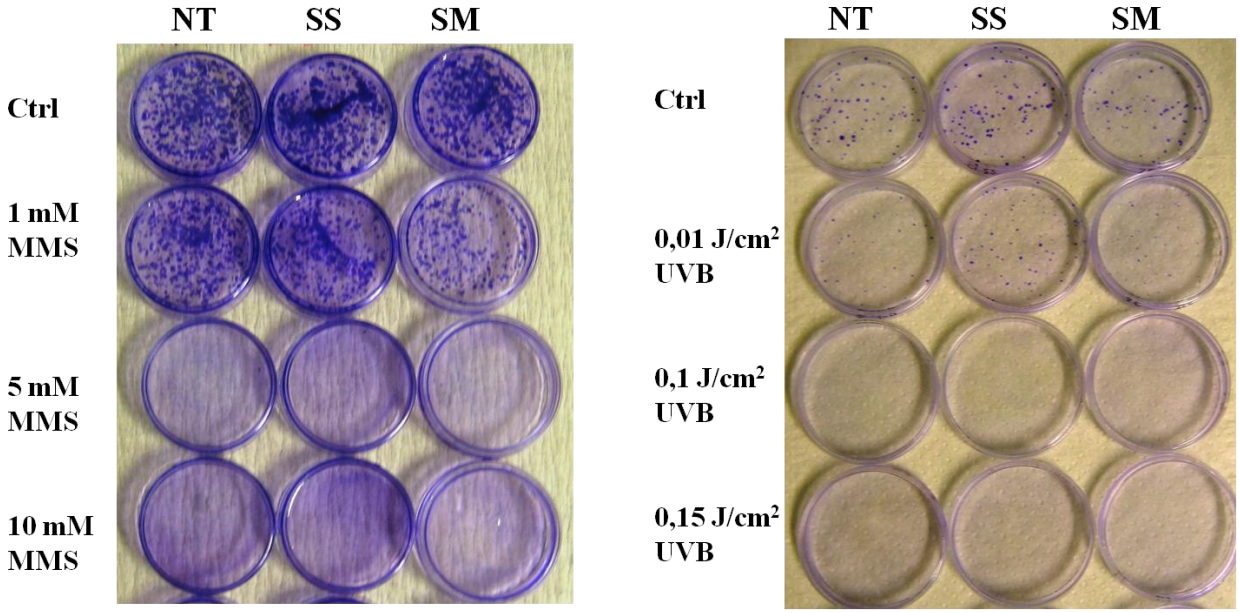

Figure 42: Representative pictures of clonogenic assay after MMS treatment or UVB irradiation in LNCaP cells, respectively. Cells were pre-treated with SS or SM for 72 h, then treated with MMS (15 min at 37°C) or UVB and re-plated at a density of 400 cells for 12 days

4. Selenium specifically protects against oxidative DNA damage

The standard version of the Comet assay, when performed under alkaline conditions, provides data for evaluating initial DNA strand breaks and alkali-labile sites (ALS) induced by exposure to different types of genotoxic stress. Figure 43 describes the important steps of Comet assay. Cells were pre-treated with or without SS and SM for 72 h and then were stressed with different agents (1). The cells were collected immediately after stress and lysed on agarose-slide support (2). The fundamental characteristic of Comet assay is to detect breaks in DNA molecule. So, to evaluate the basal DNA strand breaks we incubated slides with a buffer control (3). In order to obtain information on specific categories of DNA base lesions, several glycosylases were used to convert base lesions to strand breaks (4). These three glycosylases are Fpg, Endo III or T4 Endo V, which removed respectively oxidized purines, oxidized pyrimidines and cyclobutane pyrimidines dimers. Finally, slides were subjected to

alkaline electrophoresis step and then stained with ethidium bromide (DNA fluorescence intercalanting agent). Comet assay IV software allowed us to integrate and score Comet assay results.

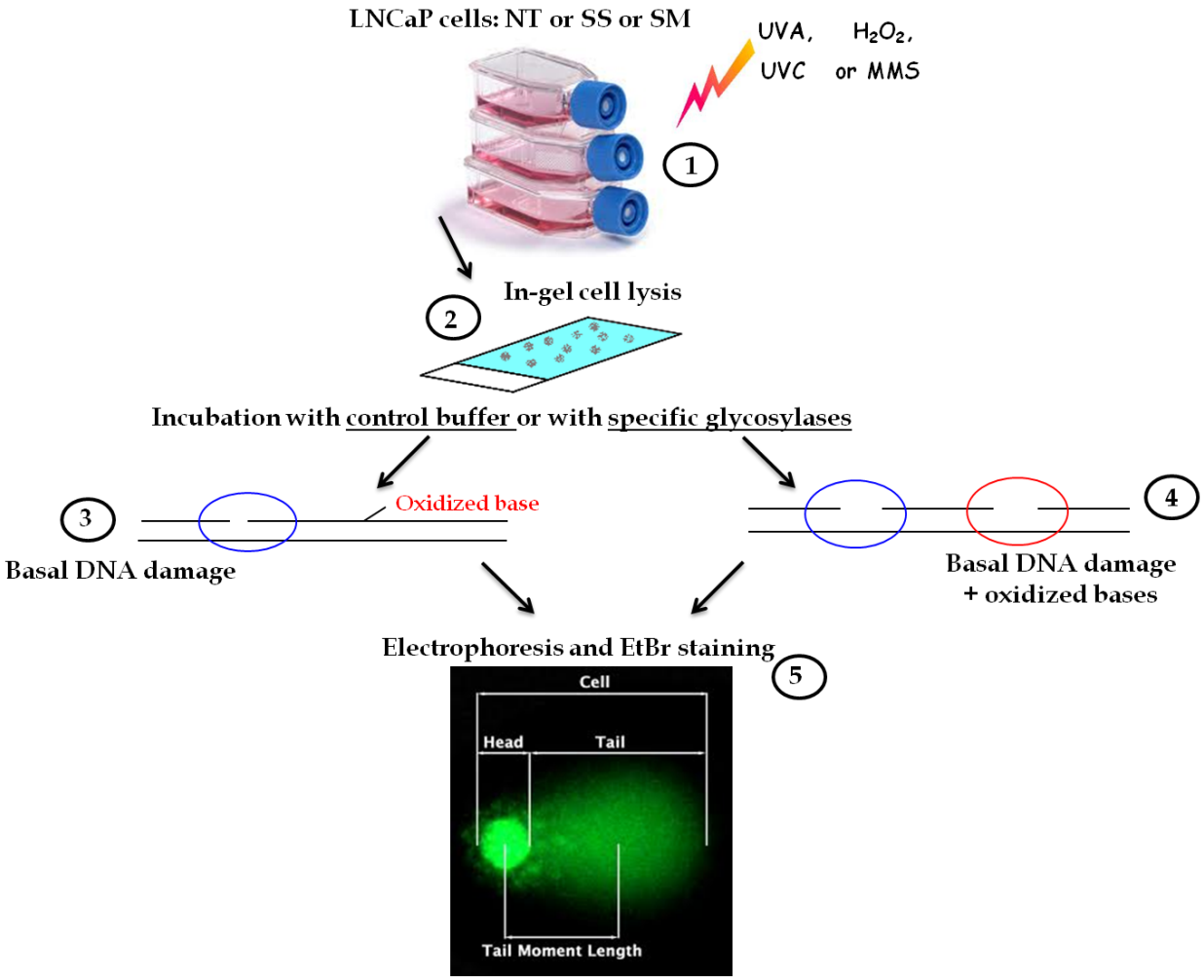

Figure 43: Summary of fundamental steps of classic and glycosylase associated-Comet assay

The Figure 44 shows an example of undamaged and damaged DNA visualized by the Comet assay. The presence of strand breaks into DNA makes it more flexible. Thus, under electrophoresis a portion of DNA leaves the nucleoid and migrates toward the positive electrode. The growing tail intensity is proportional to DNA strand breaks. The incubation with Fpg, or with another glycosylases, allows to excise the Fpg-sensitive

sites or other enzyme-sensitive site and thus to convert a oxidized purine or another base damage into strand breaks that can be detected by the increase in comet tail.

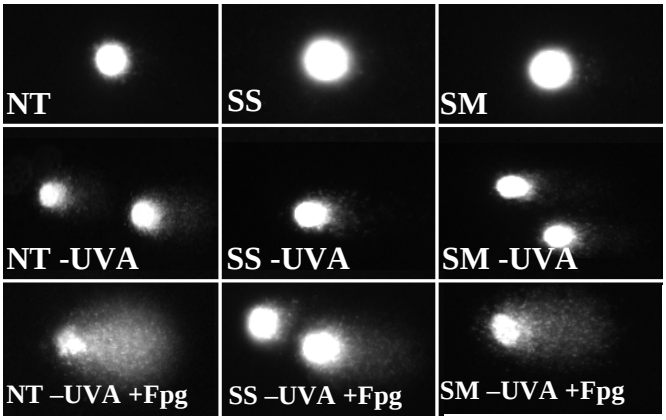

Figure 44: Photos of undamaged and damaged DNA after electrophoresis.

The Comet assay IV software analyzed different parameters linked to comet shape (head and tail). We choose to used the tail intensities because is really representative of the increase of the breaks.

The tail intensities measured for controls (NT, SS and SM) were very small and were not significantly increased upon incubation with the three repair enzymes.
The four stress agents chosen produced significant levels of DNA breaks as evidenced by increase of tail intensity.
In Figure 45 we can observe an increase of tail intensity of approximately 6-fold after exposure to 50 J/cm^2 of UVA and ~10-fold after treatment with 200 µM of H_2O_2 compared to non-treated cells. Exposure to UVA and H_2O_2 produced significant increase in the level of oxidized bases as evidenced by increase in tail intensity after Fpg digestion. Treatment with either 30 nM SS or 10 µM SM reduced the levels of DNA strand breaks induced by UVA and H_2O_2 as evidenced by significant decreases in tail intensity.

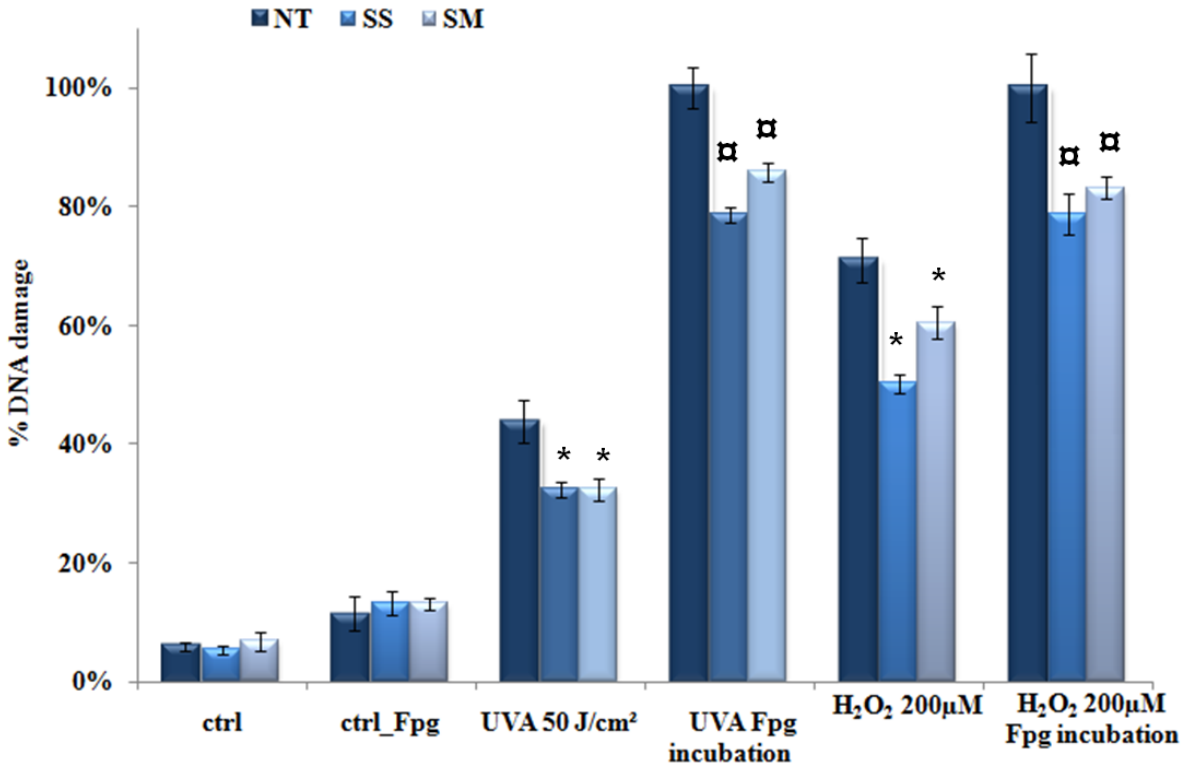

Figure 45: Selenium protects against oxidative DNA damage (* p<0.05 vs NT UVA and NT H_2O_2; ¤ p<0.05 vs NT UVA Fpg and NT H_2O_2 Fpg). LNCaP cell line was cultured with or without SS or SM for 72 h and then were irradiated with UVA at 50 J/cm² or incubated with H_2O_2 200µM (30 min on ice). The comet assay was then performed to detect SSB and ALS, and the Fpg enzyme was further used to get information on the level of oxidized purines in each sample. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

The increase of strand breaks frequency was of ~4-fold after incubation with 500 µM of MMS (Figure 46). Exposure to the monofunctional alkylating agent MMS produced significant alkylated bases as evidenced by increase in tail intensity after Fpg and EndoIII digestion (~4.5- and ~2.7-fold respectively). In contrast to response of cells to oxidative agents, no significant differences were observed in the levels of direct strand breaks after treatment with 500 µM MMS.

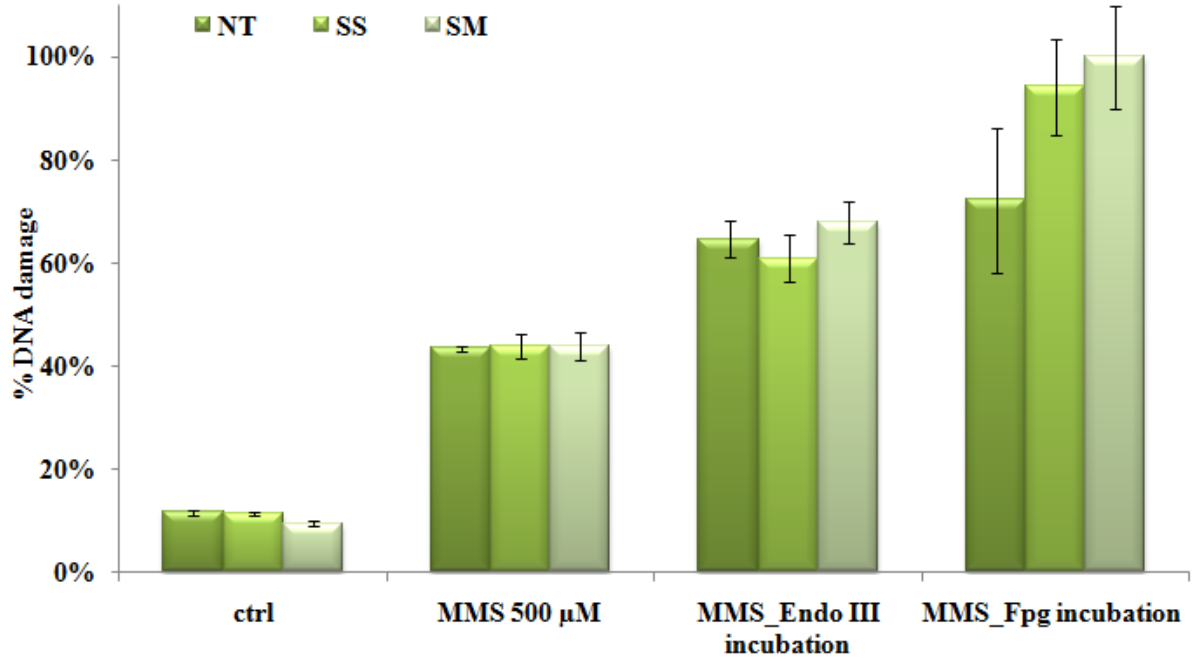

Figure 46: Selenium does not protect against alkylated DNA damage. LNCaP cell line were cultured with or without SS or SM for 72 h and then were treated with MMS 500µM (15 min at 37°C). The comet assay was then performed to detect SSB and ALS, and Fpg enzymes was further used to get information on the level of N^7MeG and Fapy, Endo III enzyme to get information about oxidized pyrimidines in each sample. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

The increase of strand breaks frequency was of ~4 fold after exposure to 10 J/m² of UVC (Figure 47) compared to non-treated cells. This UVC dose corresponds to 80% of cell viability obtained by MTT assay. In contrast to response of cells to oxidative substrates, no significant differences were observed in the levels of direct strand breaks after treatment with 10 J/m² UVC irradiation.

UVC exposure produced cyclobutane pyrimidine dimer (excised by T4 EndoV) as evidenced by the 9-fold increase in tail intensity as compared to control.

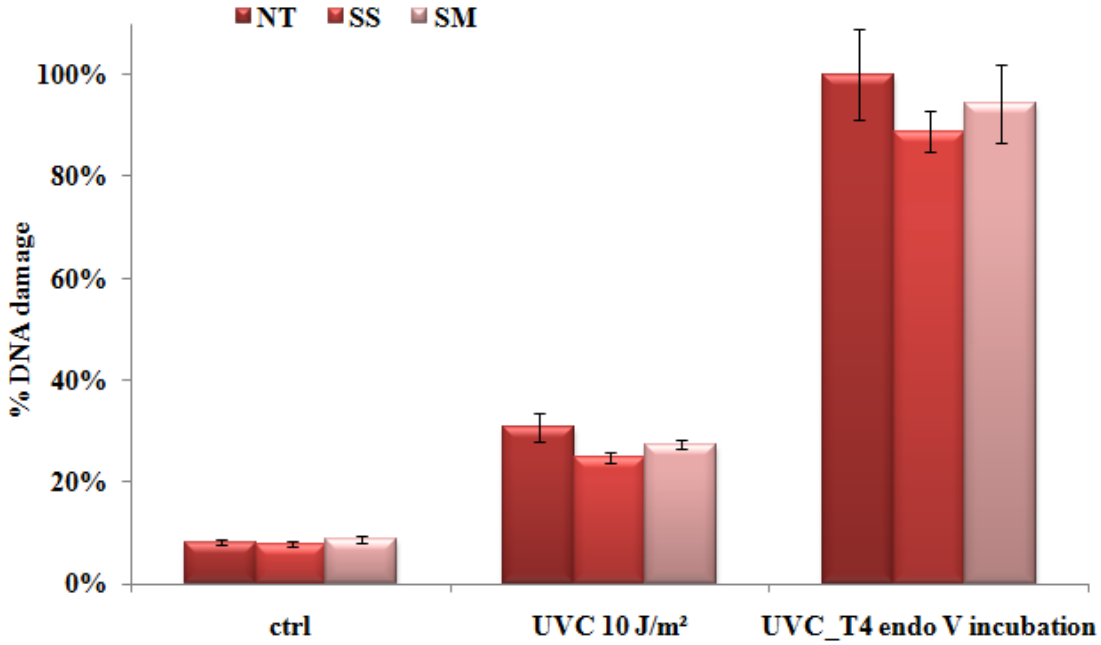

Figure 47: Selenium does not protect against CPDs DNA damage. LNCaP cells were cultured with or without SS or SM for 72 h and then were irradiated with UVC at 10 J/m². The comet assay was then performed to detect SSB and ALS, and the T4 endo V enzyme was further used to get information on the level of cyclobutane pyrimidines dimers in each sample. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

5. Formation of 8-oxoGua and CPDs upon UVA irradiation

As a complementary approach to the assessment of oxidative DNA lesions by the Fpg/Comet assay, the formation of 8-oxoGua after exposure to different doses of UVA radiation was quantified by HPLC-MS/MS analysis in NT, SS and SM cells (Figure 48). SS and SM exposure significantly decreased the induction of 8-oxoGua as compared to NT at the three applied doses (50, 100 and 200 J/cm²).

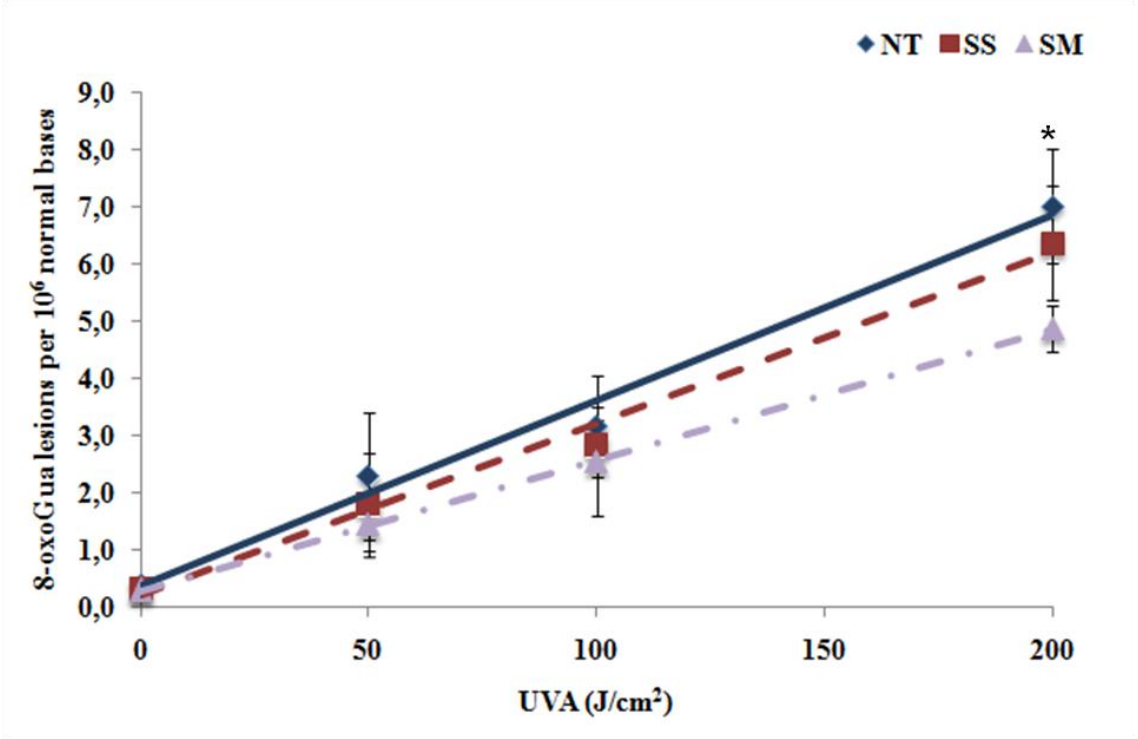

Figure 48: Dose-response curve for the formation of 8-oxoGua after UVA irradiation of LNCaP cells, detected by HPLC-MS/MS analysis. * p < 0.05 vs NT. Three biological replicates were tested in three independent experiments.

UVA radiation alone also promoted the formation of CPDs in cells, with a predominance of TT CPDs.

Figure 49, shows that at high dose of UVA irradiation we observed a slightly decreased T<>T formation in LNCaP cells pre-treated with SM.

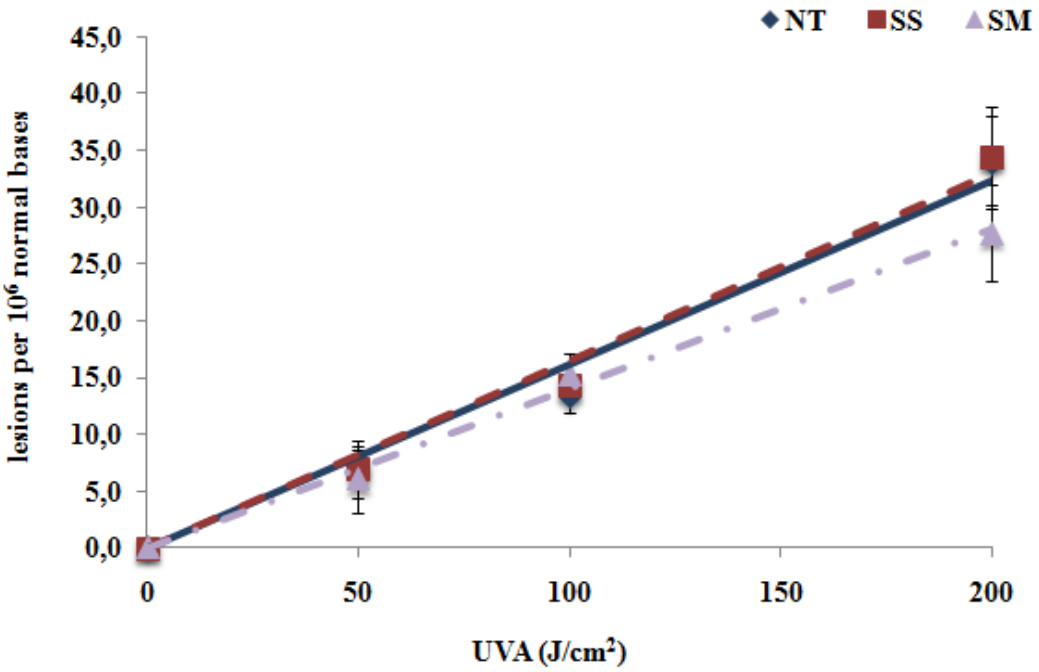

Figure 49: Dose-response curve for the formation of CPD T<>T after UVA irradiation of LNCaP cells, detected by HPLC-MS/MS analysis. Three biological replicates were tested in three independent experiments.

6. Discussion

Two selenocompounds were studied, and none was cytotoxic at the concentrations tested. Both were able to enhance viability upon exposure to UVA 50 J/cm², a typical oxidative stress-inducing agent, at the concentration of 30 nM for SS and 10 µM for SM. SS thus appears to be 300 fold more efficient than SM. This difference is likely related to the fate of the compounds in cells. Because the metabolic pathways in the body are different for the inorganic and organic forms of selenium, the absorption and bioavailability may vary. Interestingly, although sodium selenite was more potent than selenomethionine at conferring protection from UVA, it was known that SS was much more toxic to cells than was SM. This further illustrates the "two-edge" effects of selenium.

It is widely held that Se exerts many of its biochemical actions through the expression of specific selenoproteins in which Se is inserted as specific selenocysteine residues

encoded by a UGA triplet. Insertion of Se at these UGA-directed sites requires Se to be present in a chemically active form similar to selenide, and current evidence indicates that selenite is a more potent precursor of selenide than is selenomethionine (Rafferty et al., 1998). Yet, whole-body turnover studies in humans have shown that selenomethionine is re-utilized by cells more efficiently than inorganic form. This appears to arise because selenomethionine can be substituted into proteins non-specifically for methionine. When replacing methionine residues, selenomethionine appears to have no bioactivity. This mechanism appears as a trap for SM and partially explains why it is necessary to reach a concentration of SM ~300 fold higher than SS to have the same increase of selenoenzymes activity (GPx1 and TrxR). Further evidence for the increased bioactivity of selenite over selenomethionine comes from the observation that selenite, but not selenomethionine, can be converted within the cell into selenodiglutathione, a form of Se which appears able to regulate apoptosis (Rafferty et al., 1998; Wu et al., 1995). The availability and metabolism of nutritional selenocompounds showed differences in the distribution of the sodium selenite and selenomethionine among different organs (liver, kidney, pancreas and serum). In particular, SM was detected in all organs suggesting similarities with the essential amino acid methionine. Furthermore, sodium selenite was distributed more efficiently then selenomethionine in all analyzed organs (liver, kidney and serum) except pancreas (Suzuki et al., 2006b). Moreover, Rafferty at al. showed the different selenoproteins profile of primary human skin cells (fibroblast, keratinocytes and melanocytes) after 72 h incubation with selenite (Rafferty et al., 1998). These observations suggest that in the body, SS and SM may exert differential effects both in time, target organs and cell type.

The effectiveness of Se incorporation can be observed at protein level. GPx1 activity dramatically decreases under conditions of Se-deficiency and increases during Se-repletion, thus making GPx1 a useful biomarker for Se status (Sunde et al., 2009). In addition GPx1 is related to cancer. Indeed, a genetic polymorphism encodes for either proline (Pro) or leucine (Leu) at codon 198 of human GPx1. Several epidemiological studies that examined the possible association of GPx1 Pro198Leu polymorphism and

cancer had given contradictory results. Some studies reported that Leu allele was associated with an increase risk of breast cancer (Hu and Diamond, 2003), while others an overall protective effect of the Leu allele on the risk for prostate cancer (Arsova-Sarafinovska et al., 2009). This polymorphism showed a decreased response of GPx1 activity to selenium supplementation (Zhuo et al., 2009). The significantly increase of GPx1 activity in our cell line model clearly established that LNCaP cells are sensible to selenium supplementation.

In our study, the increase of transcription level of GPx1 after pre-treatment with 30 nM SS compared to NT condition attests that LNCaP cells were sensitive to selenium supplementation. Interestingly, GPx1 gene expression for SS is higher than SM. These results suggested that different forms of Se had different effects on the regulation of the amount and/or stability of mRNA of GPx1. At the protein level, GPx1 is significantly over-expressed when cells were treated with SS or SM compared to cells without selenium supplementation.

We also investigated on the protein expression of the thioredoxine reductase (TrxR). Unlike GPx1 expression, TrxR was just slightly increased after SS or SM pre-treatment. This was not unexpected since the expression of some selenoproteins in mammalian cells and tissues, such as TrxR1 (designated as housekeeping selenoproteins), is fairly insensitive to selenium status. This contrasts with others, such as GPx1 (designated as stress-related selenoproteins), are highly sensitive and are poorly expressed under conditions of selenium deficiency, which is a phenomenon known as selenoprotein hierarchy (Sunde et al., 2009). The expression of stress-related selenoproteins is dependent on the presence of selenium and their abundance correlates with the presence of mcm^5Um. On the other hand, the expression of housekeeping selenoproteins is less dependent on selenium status and their expression occurs in the presence of mcm^5U (Moustafa et al., 2001). As expected, mice and organs lacking the mcm^5Um isoform synthesize only housekeeping selenoproteins.

Finally, we also compared the activity of GPx1 and TrxR1 after selenium pre-treatment to cells without selenium supplementation. Both SS and SM supplementation enhanced

the activity of these two selenoproteins. Again it is important to underline the different concentrations used for SS and SM pre-treatment to obtain these similar results.
These findings point out clearly that pre-treatment with sodium selenite and selenomethionine increase the expression and the activity of two key selenoproteins for redox state of the cell.

To gain further mechanistic insight, we then performed several cytotoxicity assays using different kind of stress agents. Selenocompounds pre-treatment showed a protective effect on cell survival only after an oxidative stress. Moreover, if selenium was added immediately after treatment for the following 24 h, no protection was achieved against all type of stresses, even oxidative ones. These data suggest that selenium may be acting through incorporation into selenoproteins rather than by a direct antioxidant chemical action.
Cytotoxicity assays clearly lead to the conclusion that, through the antioxidant effect of selenium, the cells stressed with UVA, H_2O_2 or potassium bromate survive better in the presence of selenium. Unfortunately we could not observe a protective effect of selenium to the γ rays irradiated cells (An et al., 2007). This is because LNCaP cells are radioresistant, only with a clonogenic assay we could verify if even in these conditions selenium pre-treatment could be protective, as γ-rays induce the formation of ROS and the DNA lesions derived from them.
The selenocompounds tested were not protective against neither MMS, UVC- nor UVB-induced cytotoxicity, indicating that Se supplementation supplied protection only against ROS-induced cytotoxicity. These data on human cells are in contrast with other obtained in rodent cells. The effect of non-toxic concentration of another selenocompound, diphenyl diselenide (DPDS), protects Chinese hamster V79 cells against MMS and UVC (Rosa et al., 2007). DPDS is an important compound used as an electrophilic agent in the synthesis of a range of pharmacologically active organic selenium compounds and they are also mimetics of native GPx enzyme (Sausen de Freitas et al., 2010). These contradictory results emphasize the importance of the chemical form of Se.

Our cytotoxicity experiments with Cis-Pt did not show a protection by selenium compounds. However, a previous study demonstrated that physiologyically non-toxic doses of ebselen provided protection against cisplatin-induced nephrotoxicity (Baldew et al., 1990). In contrast to most selenium compounds, the selenium moiety of ebselen (seleno-organic compound showing glutathione peroxidase-like activity) is not available for incorporation into proteins and, moreover, ebselen is not converted into hydrogen selenide. Chemoprotection against cis-platin-induced toxicity can be achieved through nucleophilic agents, which are capable of react with cisplatin and its hydrolysis products (Baldew et al., 1989).

In contrast with other studies that use synthetic compounds of selenium we did not observe any beneficial effect of pre-treatment on cell survival after stress with MMS, UVB, UVC and the two chemopreventive compounds. These findings proved that the selenocompound forms but also the cell line are fundamental to observe an influence of selenium supplementation and a consequent protection against exogenous agents.

In addition, to cellular lethality, damage to the genome was investigated as a key factor toward cancer prevention. The extent of DNA damage was investigated by two complementary approaches: the Comet assay and HPLC-MS/MS assessment of 8-oxoGua levels. Although the Comet assay is sensitive and qualitative, it is not adequate to quantitatively assess specific base damage, which was obtained by HPLC-MS/MS analysis. We observed that Se can modulate the induction of damage after exposure to DNA damaging agents. This was demonstrated for the induction of strand breaks induced by UVA and H_2O_2, as well as 8-oxoGua resulting from UVA irradiation. The significant decrease in 8-oxoGua formation in Se-treated cells upon UVA irradiation was confirmed only after HPLC-MS/MS analysis. These results are therefore consistent with the cell survival data indicating that Se compounds are efficient at protecting cells from oxidative damage.

The absence of protection against other types of stress, either alkylating agent or radiation (UVC), emphasizes that SS and SM were only able to protect against oxidative stress and may only be regulators of intracellular redox status.

Rosa et al. also showed that diphenyl diselenide supplementation reduced DNA damage after MMS and UVC treatment using Comet assay (Rosa et al., 2007). These observations indicate that the protective efficiency of Se derivatives strongly depends on the chemical nature of the stress and further studies should consider including other cell types.

In conclusion, the interesting cytotoxicity results have highlighted that a pre-treatment with SS or SM confers a significantly increase of cell survival in response to an oxidative stress. Even more interesting is the important decrease of DNA damage by selenium supplementation still after oxidative stress. We believe that the action of selenium is mediated by the activity of antioxidant enzymes leading to a reduction of ROS. More complex cellular signaling mechanisms and biological responses could also be involved. For this purpose, the second part of the work will be devoted to the effect of selenium pretreatment on DNA repair.

Chapter 3 – Evaluation of DNA repair capacity after selenium supplementation in LNCaP cells

In the previous chapter, we have justified the importance of the chemical form and concentration of selenocompounds as well as the cellular model used. We also showed that selenium was able to protect cells from lethality after stress, particularly oxidative ones. Se was also showed to be able to reduce the formation of DNA damage immediately after UVA irradiation and H_2O_2 incubation. In particular, after these treatments we observed a significantly decrease of formation of strand breaks and oxidized purine.

These findings gave us a more specific idea about the possible molecular mechanism of selenium supplementation that may be related to damage to the genome.

At present, the most part of selenium studies are epidemiological or clinical ones that did not investigate a lot about the underlying molecular mechanism. In particular, it is not clearly known whether decrease in DNA damage is associated with an improvement of the DNA repair systems response.

Consequently, this chapter will be devoted to the study of DNA repair capacity in LNCaP cells pre-treated with selenocompounds.

First, we investigated the rate of DNA repair by kinetic studies. We carried out Comet assay at different time to determine the repair rate of different type of damage.

The different repair capacity between cells with or without selenium pre-treatment led us to determine the gene level expression of several repair enzymes involved in BER. The results compared the basal level gene expression and the induction of the transcription after UVA radiation. The interesting results obtained allowed us to also evaluate the protein expression level of some enzymes.

Finally, we used two complementary assays to evaluate the excision capacity of protein extracts. We examined the effect of Se on DNA repair capacity using a modified-Comet assay to measure excision activity of the protein extracts on genomic

DNA and the oligonucleotide cleavage assay on biochip (ODN) to quantify simultaneously several glycosylases activities contained in a nuclear cell extract.

1. Kinetic study of DNA damage repair by Comet assay

A kinetic study by Comet assay was carried out to follow the repair of DNA damage produced by different DNA damaging agents. Cells were pre-treated or not with selenocompounds for 72 h and irradiated with UVA (10 J/cm²). The irradiation dose corresponds to 80 % of cell viability in non-irradiated cells determined during MTT assay. After irradiation cells were collected at different time for DNA damage analysis.

Figure 50 shows the repair kinetics of strand breaks. The strand breaks measured include single, double stands breaks, and ALS that under alkaline working condition are converted into strand breaks. We observed that the rate of repair was the same for the three conditions. So, SS and SM pre-treatments do not influence the repair of DNA strand breaks.

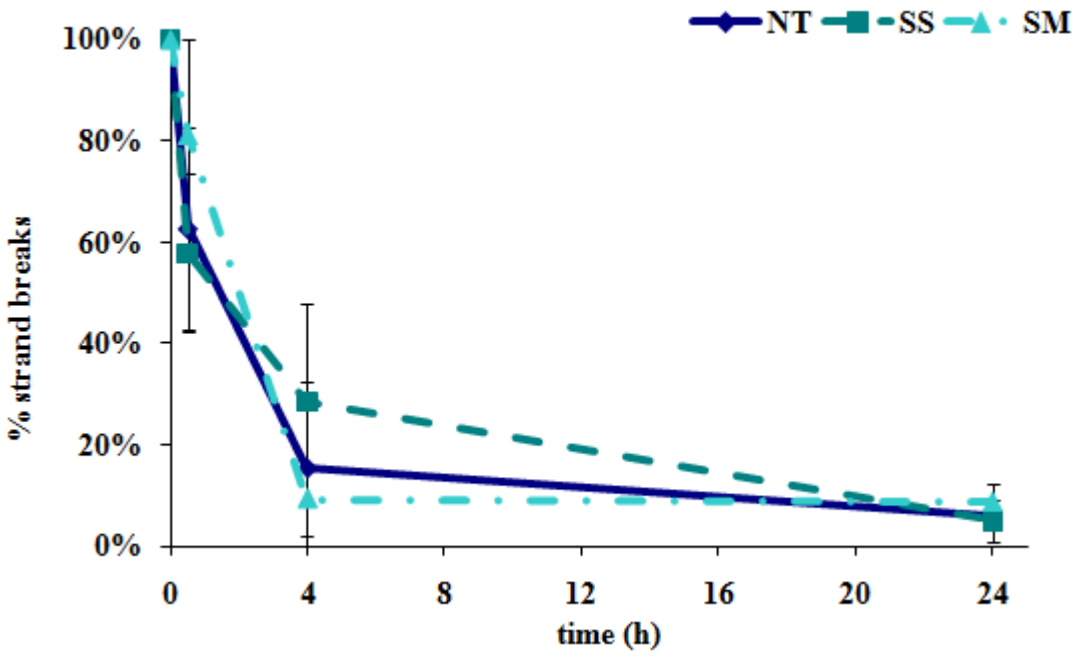

Figure 50: Repair kinetic of DNA strand breaks. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were irradiated with UVA at 10 J/cm². Cells were collected at different time after irradiation (30 min, 4 and 24 h) and we carried out a comet assay. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

Comet assay also gave us the possibility to determine the repair kinetics of oxidized bases. Additional information on specific categories of DNA lesions could be obtained through quantification of lesions recognized by the repair enzyme Fpg which catalyzes the excision of oxidized purines and Fapy derivates.

The 100% value corresponds to the maximal damage at time zero, immediately after irradiation. The other values are calculated respectively to this one for the different conditions. We showed that the rate of repair of Fpg-sensitive sites is significantly faster in cells pre-treated with SS or SM 4 h after UVA irradiation compared to NT (Figure 51).

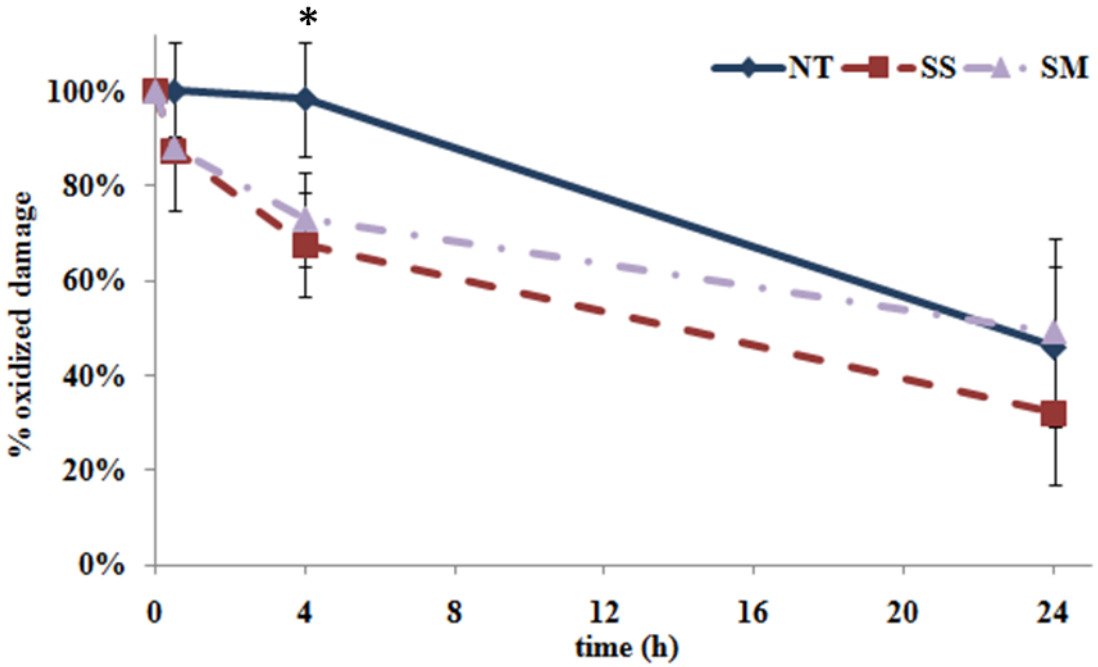

Figure 51: Repair kinetic of oxidized purines after UVA irradiation. * p < 0.05 compared to NT 4h. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were irradiated with UVA at 10 J/cm². Cells were collected at different time after irradiation (30 min, 4 and 24 h) and we carried out a comet assay with a step of Fpg incubation for 30 min at 37°C. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

To follow the repair kinetics of 8-oxoGua, we also used a photosensitizer (Riboflavin) to irradiate cells with UVA 10 J/cm². Riboflavin exerts its oxidizing power only after being excited by a source of visible light or UV. In our case, we have chosen to use a

low dose of UVA to activate the riboflavin, to avoid a significant formation of dimeric photoproducts generated by UV. Moreover, Douki et al. showed that the predominant product of riboflavin mediated photosensitization is 8-oxoGua (Douki and Cadet, 1999). Figure 52 confirms that the repair of 8-oxoGua is faster in cells pre-treated with selenium 4 h after the irradiation.

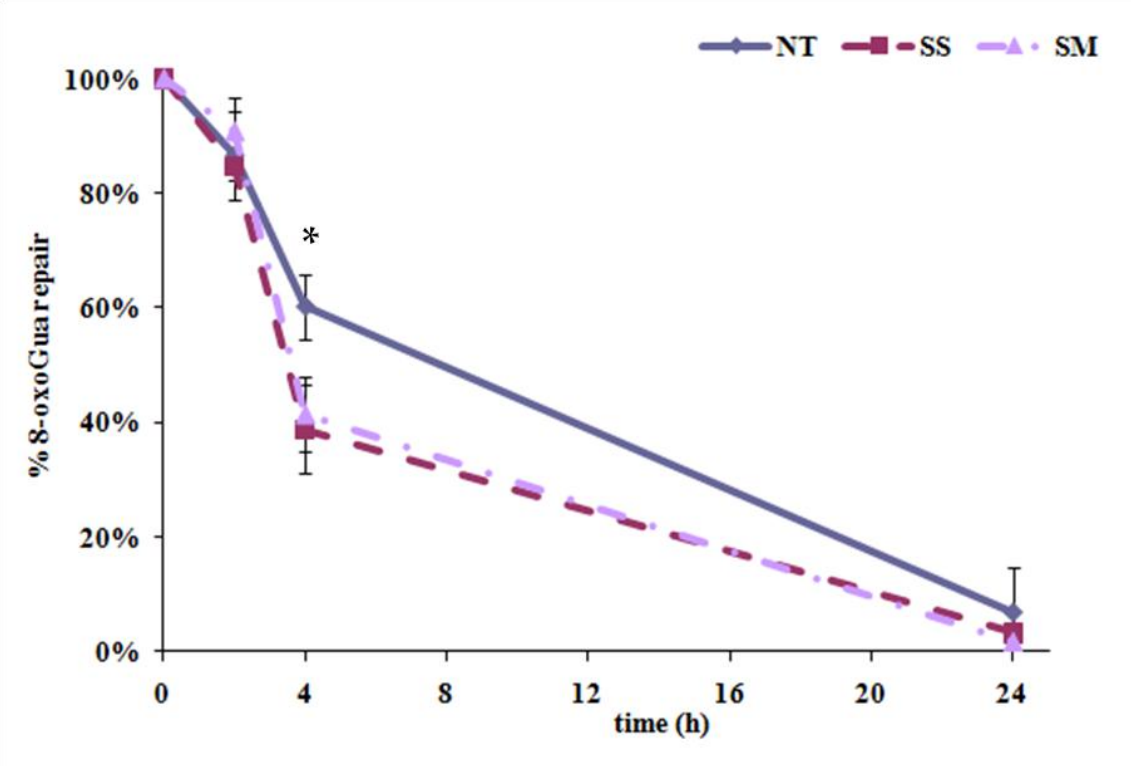

Figure 52 : Kinetic repair of 8-oxoGua after Riboflavin incubation and UVA irradiation. * p < 0.05 compared to NT 4h. LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h. Cells were incubated 20 min at 37°C with Riboflavin 1 µM and then were irradiated with UVA at 10 J/cm². Cells were collected at different time after irradiation (30 min, 4 and 24 h) and we carried out a comet assay with a step of Fpg incubation for 30 min at 37°C. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

2. BER-associated gene expression profile

The faster 8-oxoGua repair in cells pre-treated with SS and SM compared to NT suggested a increased base excision repair capacity. This led us to measure the gene expression of several repair enzymes involved in the BER system by qPCR. The housekeeping gene used as a reference was S18. The genes analyzed are APE1 that is

an AP endonuclease that cleaves abasic sites; NTHL1 removes damaged DNA at cytosines, thymines and guanines; UNG excises uracil residues from the DNA; PARP1 plays a role in repair of single-strand DNA breaks; OGG1 excises DNA at 8-oxoG and Fapy residues; MYH removes the adenine inappropriately paired with 8-oxoGua from the DNA backbone; NEIL recognizes and removes oxidized pyrimidines (such as thymine glycol and 5-hydroxyuracil); XRCC1 is involved in the efficient repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents.

At first, we compared the basal level of gene expression. Only OGG1 was found to be over-expressed for the SS condition (Figure 53).

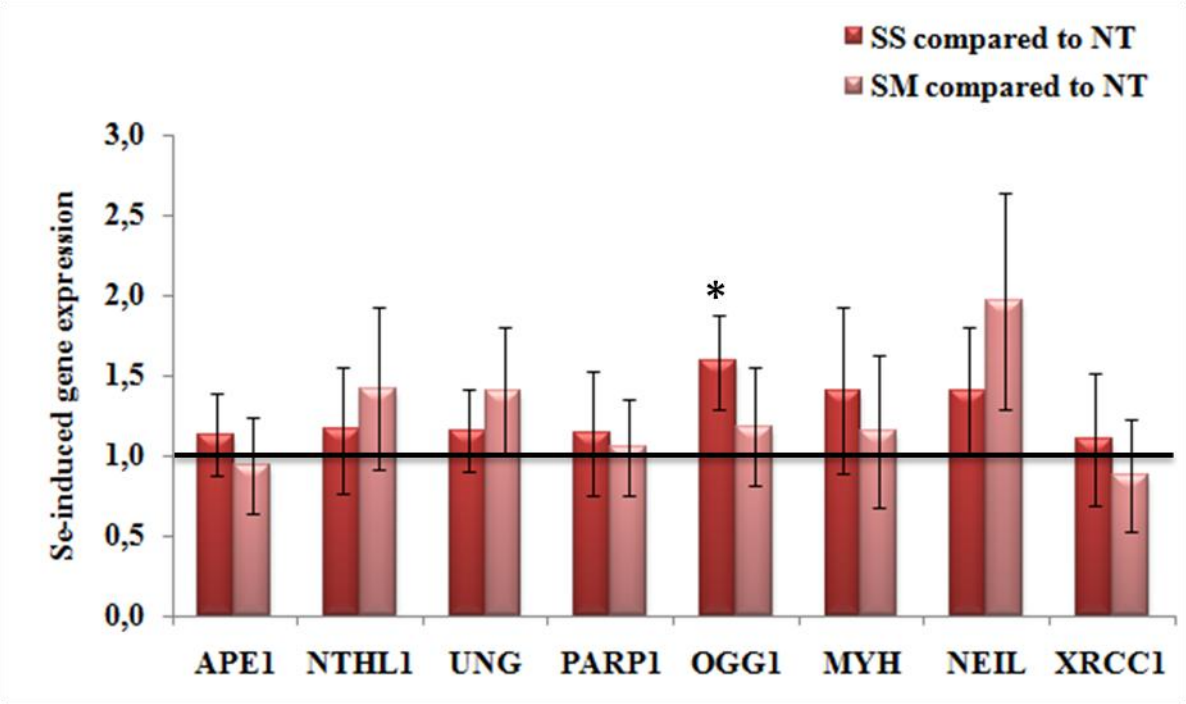

Figure 53: BER-associated gene expression profile at basal level in cells pre-treated with SS or SM compared to NT. * p < 0.05 vs NT. LNCaP cells were cultured for 72 h with or without SS or SM. Total RNA was extracted and subsequently reverse-transcribed. A total of 20 ng of corresponding cDNA were used to detect specific gene expression using real-time qPCR. Absolute gene expression in SS or SM conditions compared to NT cells was calculated using REST. Three biological replicates were tested in triplicate in three independent experiments. The mean of the corresponding expression ratios was calculated, and a Student's t-test was performed.

The cells were pre-treated or not with SS and SM for 72 h, irradiated with UVA 10 J/cm² and RNA was collected 4 h after stress. The kinetic results showed that 4 h after irradiation ~40% of oxidized purines were repaired. We then investigated the modulation of BER genes to oxidative stress. Figure 54 shows the ratio between the gene expressions of UVA-irradiated cells and the non-irradiated ones. The UVA radiation significantly decreases the gene expression of several enzymes involved in the recognition and excision of the damage. Yet, no difference was observed in gene expression between cells treated or not with selenocompounds.

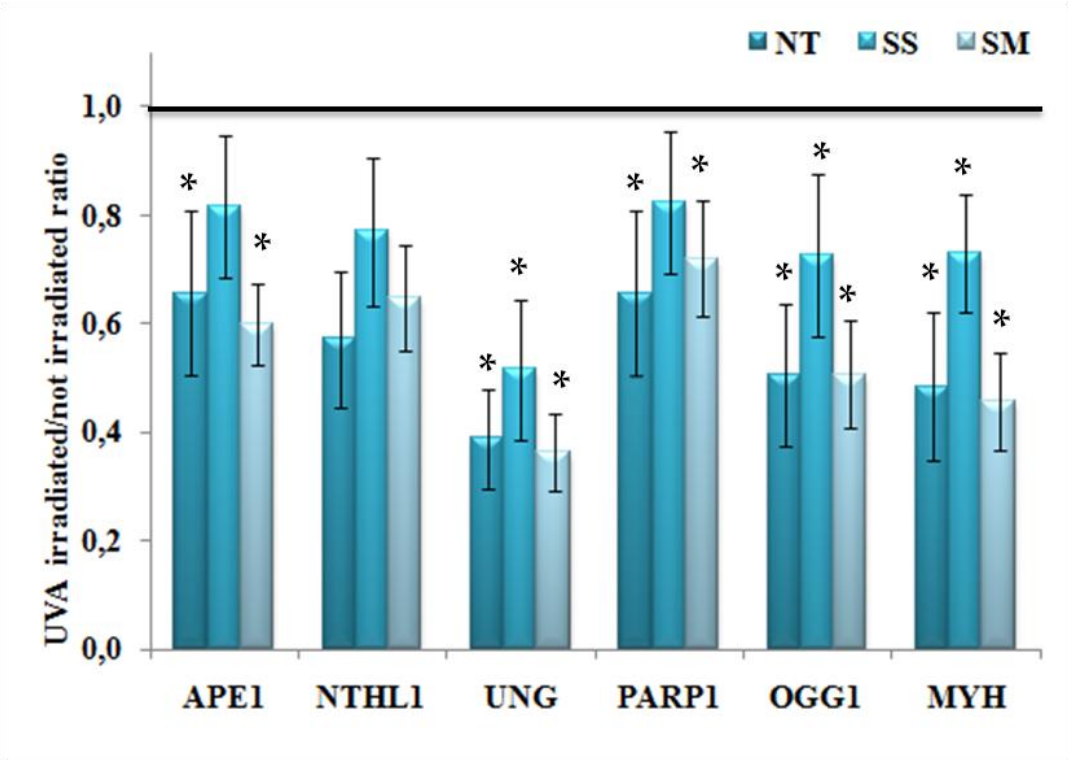

Figure 54: Effect of UVA on BER associated genes. * p < 0.05 vs non-irradiated. LNCaP cells were cultured for 72 h with or without SS or SM and then irradiated with UVA at 10 J/cm² and cells were collected 4 h after irradiation. Total RNA was extracted and subsequently reverse-transcribed. A total of 20 ng of corresponding cDNA were used to detect specific gene expression using real-time qPCR. Gene expression for NT, SS or SM conditions compared to non-irradiated cells. Three biological replicates were tested in triplicate in three independent experiments. The mean of the corresponding expression ratios was calculated, and a Student's t-test was performed.

3. Protein expression

The following step was to evaluate the protein expression, by Western Blotting, after UVA exposure of cells pre-treated or not with SS and SM. Cells were collected 24 h after irradiation.

We analysed the protein expression of APE1 and hOGG1. Figure 55 presents a picture of gel analysis performed. These two enzymes are involved in the first step of the recognition and excision of 8-oxoGua. The two proteins are together recruited to nuclear speckles in UVA-irradiated cells. For this reason, we carried out the assay only with protein nuclear extract. Unlike the western blot analysis of GPx1 and TrxR, we did not used β-actin as control that is a cytoplasmatic protein, but we used TBP (TATA box binding protein) antibody that has a nuclear localization.

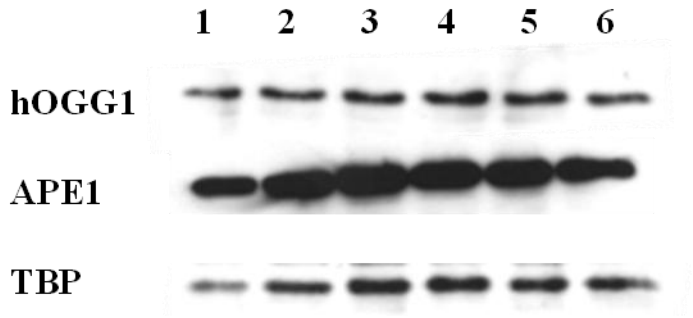

Figure 55 : Representative picture of Western blot analysis performed with nuclear extracts from NT, SS orSM conditions irradiated or not with UVA at 10 J/cm². Line 1: NT; line 2: SS; line 3: SM; line 4: NT UVA, line 5: SS UVA; line 6: SM UVA. The relative abundance of each targeted protein are displayed in picture with the corresponding membrane-revealed TBP controls.

Figure 56 shows that, at the basal level, hOGG1 is slightly over-expressed in SS and SM conditions compared to NT. This is the same trend than for gene expression in SS pre-treated cells. Moreover, we also observed an increase of hOGG1 in cells pre-treated with selenomethionine while gene expression was not modified. In contrast, content in APE1 protein was not modified for the two conditions. Similarly, the qPCR analysis showed that selenium supplementation did not induce an increase of mRNA level of APE1.

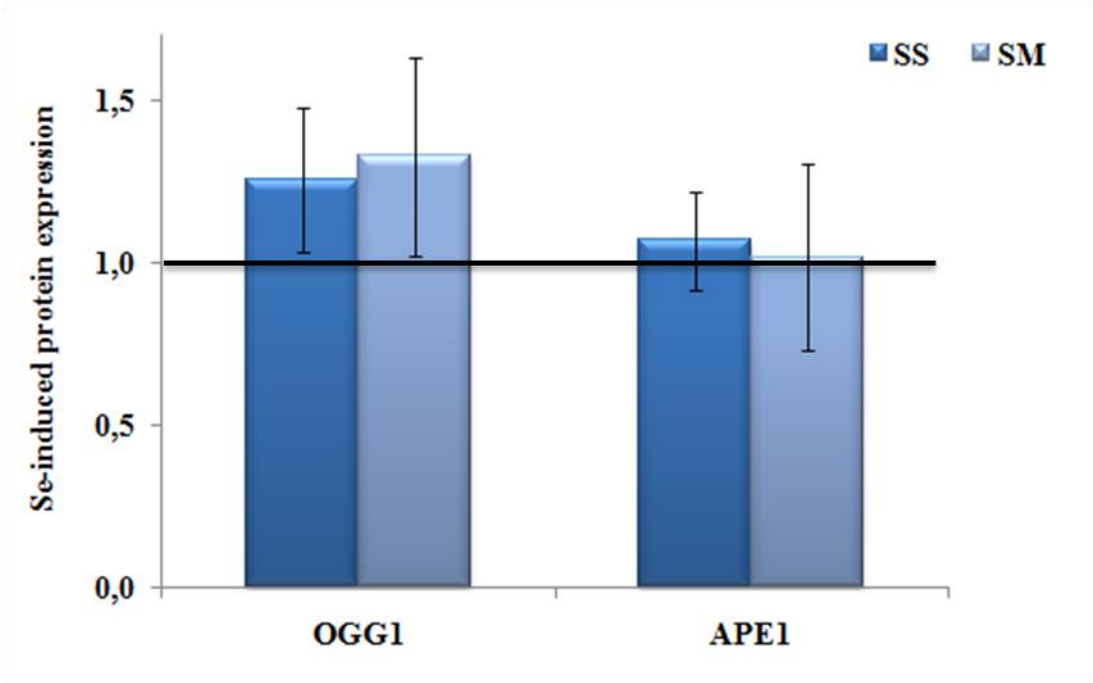

Figure 56: Protein expression levels of hOGG1 and APE1 after SS or SM pre-treatement. Cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were collected. Total protein extracts were prepared and western blot analysis was performed. Three biological replicates were tested in triplicate in three independent experiments.

Then, we compared the expression level of hOGG1 and APE1 after UVA irradiation with 10 J/cm^2 24 h after the stress. Contrary to qPCR data, hOGG1 protein was found to be over expressed after stress compared to the basal state, as shown in Figure 57. Moreover, selenium pre-treatment slightly over-expressed hOGG1 compared to NT conditions after irradiation.

APE1 expression after UVA radiation was unmodified in NT condition, and slightly decreased for SS and SM.

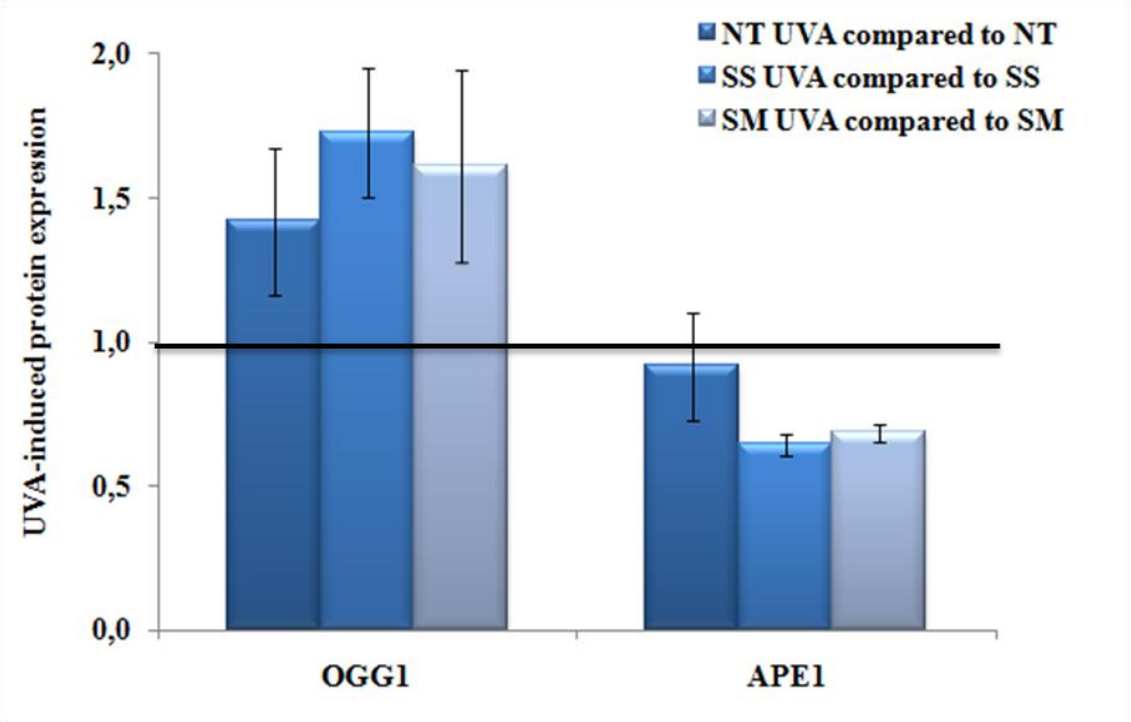

Figure 57: **Protein expression levels of hOGG1 and APE1 in LNCaP cells upon UVA irradiation.** Cells were pre-treated with 30 nM SS or 10 µM SM for 72 h and then were irradiated with UVA 10 J/cm². Cells were collected 24 h after the irradiation. Total protein extracts were prepared and western blot analysis was performed. Three biological replicates were tested in triplicate in three independent experiments.

4. Selenium supplementation increases 8oxoGua excision capacity

4.1 Assessment of cellular DNA repair capacity in protein extracts

To study the effect of Se supplementation on DNA repair, we used a modified Comet assay-based approach, which assessed the ability of the repair enzymes present in cellular extracts to incise the DNA of damaged cells used as substrate and cause single-strand breaks. The main steps are summarized in Figure 58. We prepared different damaged substrates and the respective controls; we plated LNCaP cells and then treated either with Riboflavin 1 µM + UVA 10 J/cm², 200 µM H_2O_2, 500 µM MMS or 10 J/m² UVC (1). The cells were collected and analyzed on agarose-slide support. In parallel, we pre-treated cells with 30 nM SS or 10 µM SM for 72 h and then we collected cells to extract total proteins (2). Slides with lysed substrates were incubated with protein extracts for NT, SS or SM conditions and also with specific

purified repair enzymes and their buffer (3). Like classical Comet assay we carried out an electrophoresis step and stained slide for image analysis (4).

The control samples were used as a negative control to evaluate the effect of buffer and Fpg incubation, and also the absence of nuclease in the protein extracts. As a positive control, we evaluated the glycosylase sensitive-sites values that were significantly higher than the three cell extracts values, except for MMS.

The initial steps of the repair processes include the sensing of the additional DNA damage, followed by the excision of the damage, resulting in single-strand breaks. The increase in the tail intensity after incubation of the damaged substrates with the cell extracts is an indication of enhanced repair capacity of the enzymes involved (5). The specificity of the assay was provided by the nature of the lesions present in the substrate. Using substrates with distinct classes of damage, both BER and NER could be assessed.

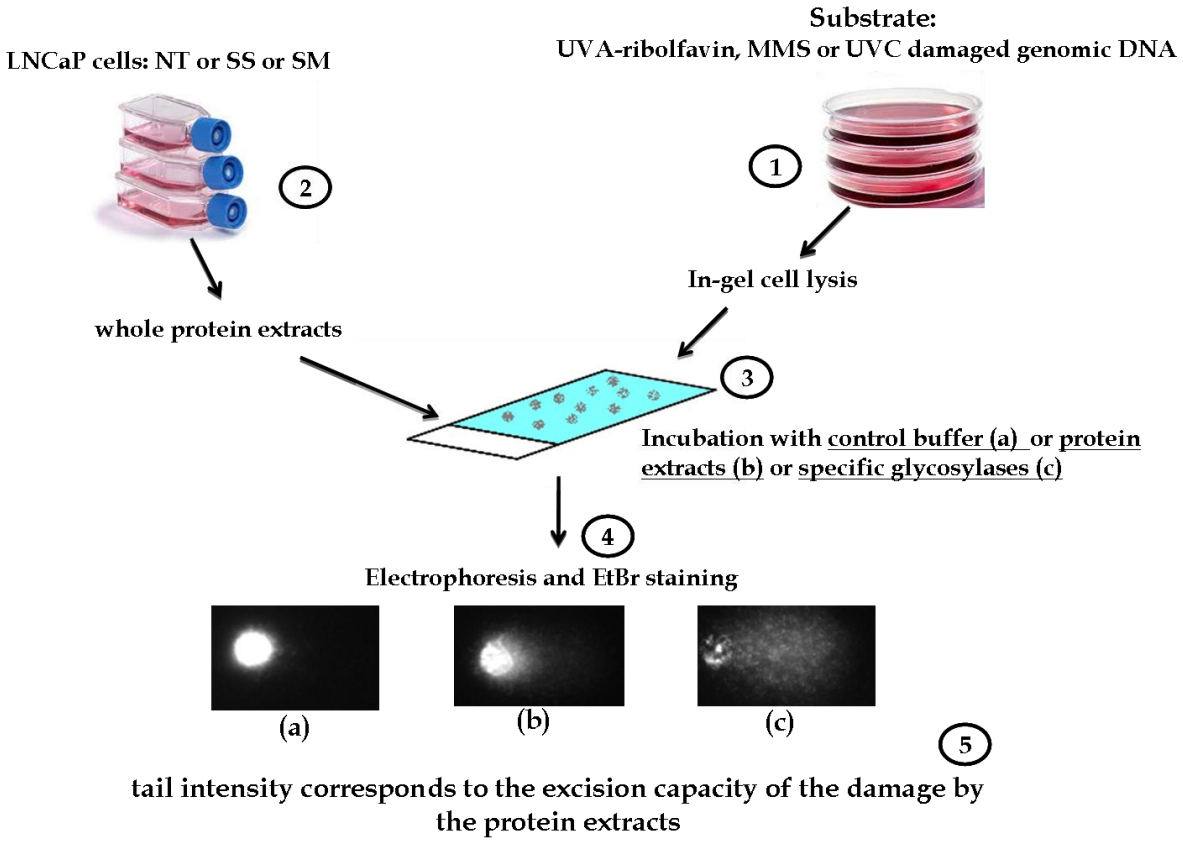

Figure 58: Summary of fundamental steps of modified-Comet assay approach for DNA repair

4.1.2 Validation of substrates by LC-MS/MS

To validate the substrate quality we collected the damaged cells and determined the level of DNA damage by HPLC-MS/MS. Figure 59 and Figure 60 show the amount of lesions induced by irradiation. These findings confirm that the irradiation induced an large amount of DNA damage, namely 8-oxoGua upon UVA-Riboflavin photosensitization, and CPDs and (6-4) PP upon UVC irradiation.

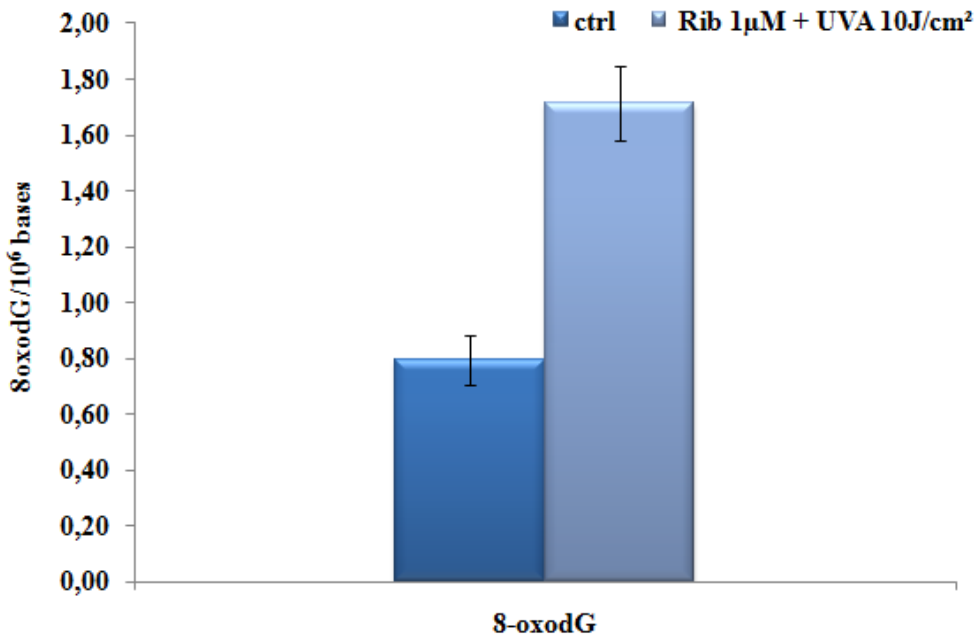

Figure 59: LC-MS/MS analysis of Comet assay substrate modified by Riboflavin incubation and UVA irradiation. . LNCaP cells were plated 72 h and were incubated 20 min at 37°C with Riboflavin 1 µM and then were irradiated with UVA at 10 J/cm². Cells were collected immediately after treatment and DNA was extracted and hydrolysed to detect the nucleosides by LC-MS/MS analysis and quantify the amount of 8-oxodG.

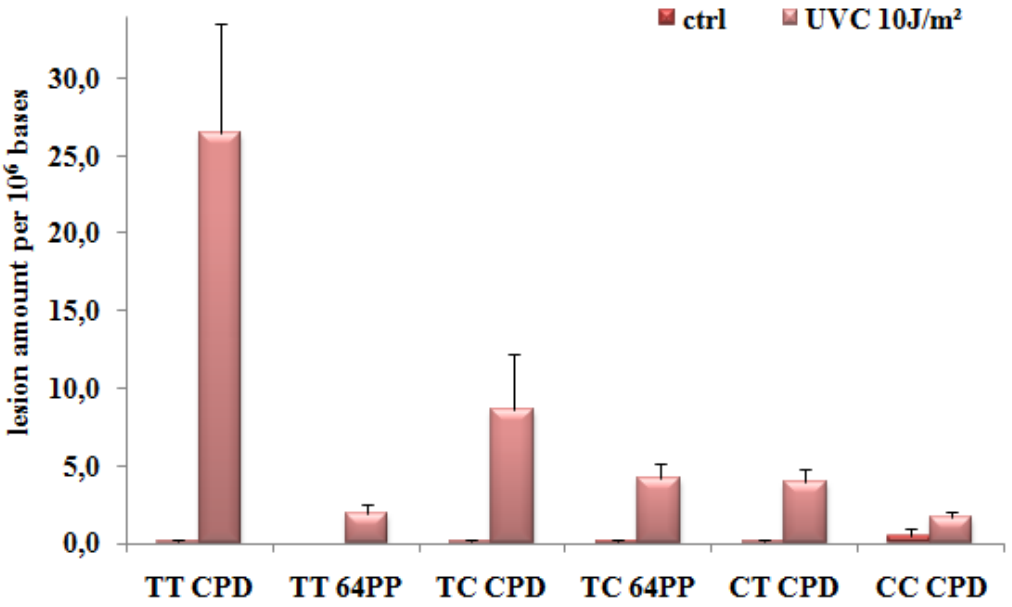

Figure 60: LC-MS/MS analysis of Comet assay substrate modified by UVC irradiation. LNCaP cells were plated 72 h then were irradiated with UVC at 10 J/m². Cells were collected immediately after treatment and DNA was extracted and hydrolysed to detect the nucleosides by LC-MS/MS analysis and quantify the amount of pyrimidines dimers and photoproducts.

4.1.2 Modified Comet assay-based approach results

DNA tail intensities for the oxidized substrate, measured following incubation with all three cell extracts (NT, SS, SM) were significantly higher compared to incubation in buffer alone (Figure 61). The respective ratios were of 1.3-, 2.0- and 1.9-fold. In addition, protein extracts from cells pre-treated with SS and SM exhibited a significantly higher excision potential (30.5 ± 4.3 and 29.9 ± 3.3 respectively) than the NT LNCaP cells (20.1 ± 3.3, $p<0.05$). Fpg sensitive-sites values (46.5±4.0) were significantly higher than the three cell extracts values.

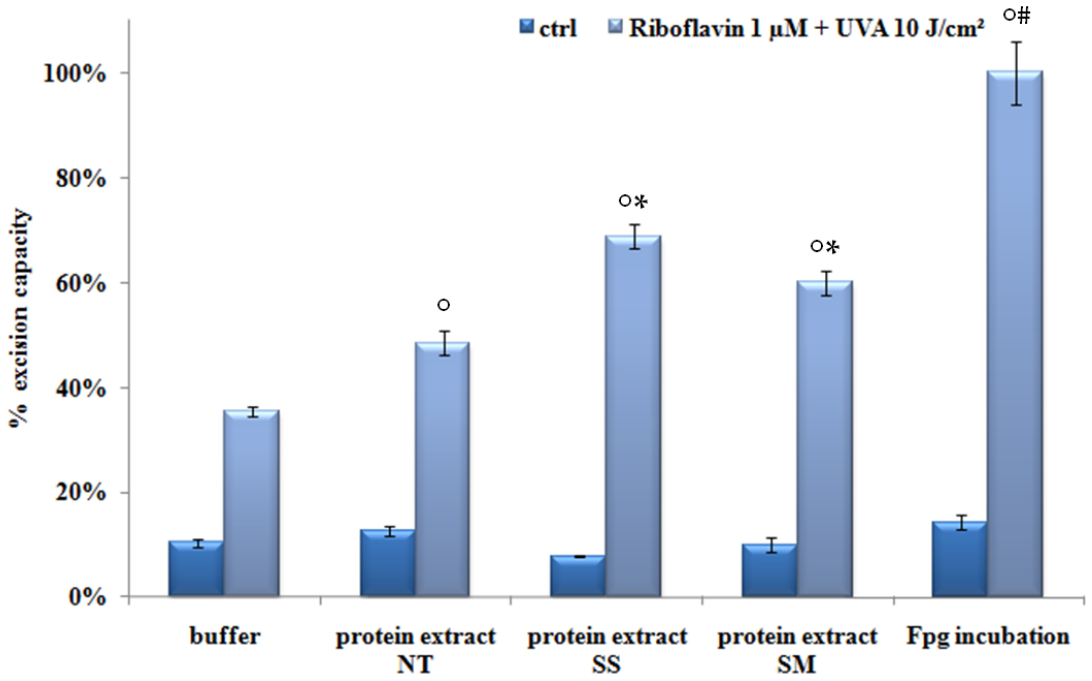

Figure 61 : Selenium supplementation increases 8oxoGua excision capacity of cell extracts on damaged substrates. °p<0.05 vs buffer; * p<0.05 vs NT cell extract, # p<0.05 cell extract. LNCaP cells were plated 72 h and were incubated 20 min at 37°C with Riboflavin 1 µM and then were irradiated with UVA at 10 J/cm². Cells were collected immediately after treatment and used as substrate for a comet assay. On the other hand, LNCaP cells were pre-treated with 30 nM SS and 10 µM SM for 72 h. Total protein cell extracts were prepared, and substrates were incubated for 30 min at 37°C to analyze 8oxoG excision capacity on damaged genomic DNA. The excision capacity (%) of the extracts was normalized to the Fpg enzyme value that was considerd the 100% of excision capacity. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

A second DNA substrate corresponding to MMS-treated cells was also used in the repair assay. Unlike the results obtained with UVA/Riboflavin damaged DNA substrate, there were no significant differences in repair capacity for the protein extracts obtained from the SS and SM treated cells as compared to the NT control cells (Figure 62). It is interesting to note that the activities of the positive controls (Endo III and Fpg) were lower compared to those of cell extracts. This can be explained by the fact that MMS generates a variety of alkylated bases that are not all substrate for these two glycosylases.

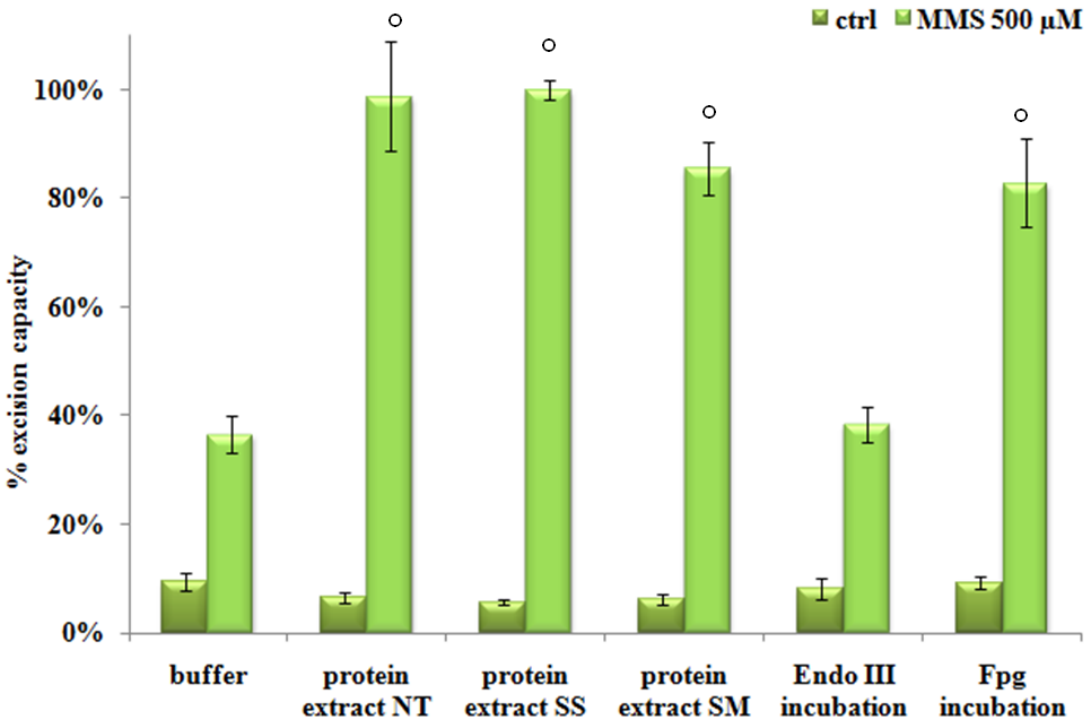

Figure 62: Excision capacity of cell extracts on MMS damaged substrates. ° p<0.05 vs buffer. LNCaP cells were plated 72 h and were incubated 15 min at 37°C with 500 µM MMS. Cells were collected immediately after treatment and used as substrate for a comet assay. On the other hand, LNCaP cells were pre-treated with 30 nM SS and 10 µM SM for 72 h. Total protein cell extracts were prepared, and substrates were incubated for 30 min at 37°C to analyze excision capacity on damaged genomic DNA. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

A similar trend was obtained for UVC-irradiated substrates (Figure 63). The DNA tail intensities (%) for all three cell extracts (NT, SS, SM) was significantly higher compared to the tail intensity of the substrate treated with the buffer. However, no differences were found among the activities of the three extracts. The efficiency of T4 Endo V under the condition used was 2.5-fold higher than the activity detected in the cell extracts.

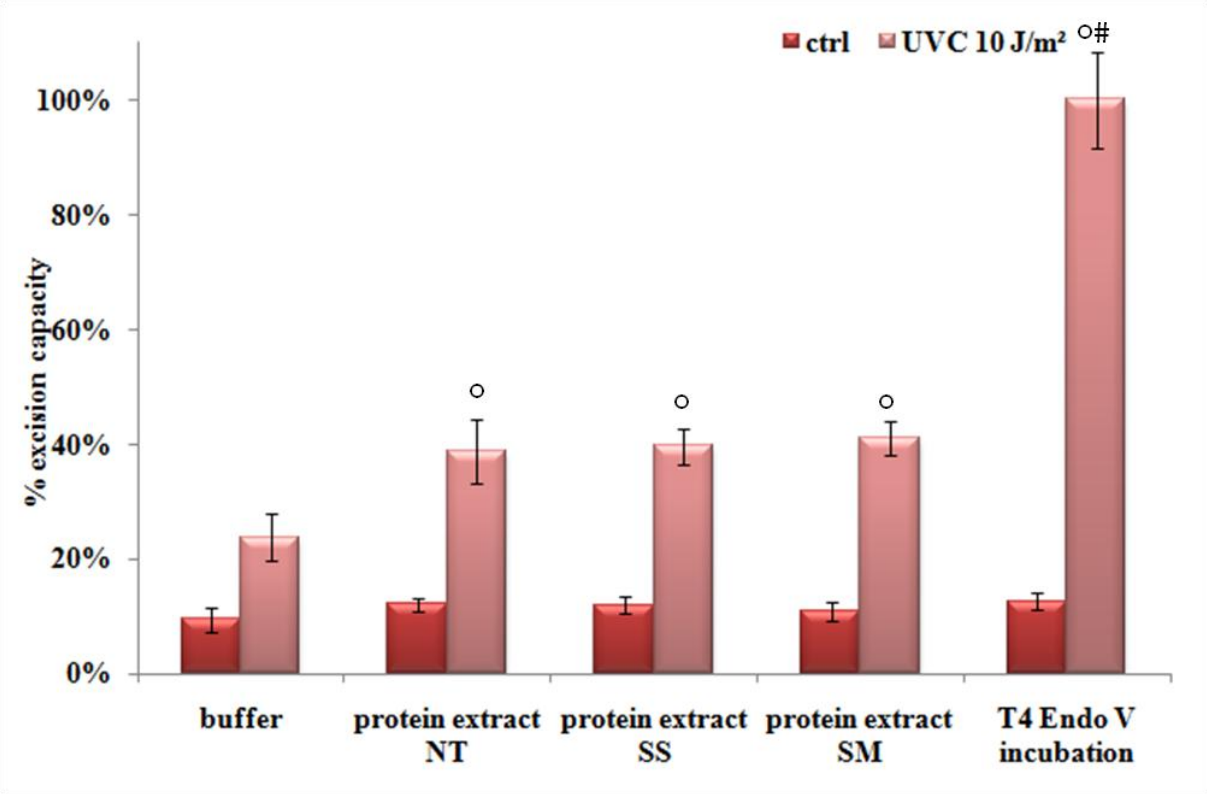

Figure 63: Excision capacity of cell extracts on UVC damaged substrates. ° p<0.05 vs buffer; # p<0.05 cell extract. LNCaP cells were plated 72 h and were irradiated with UVC at 10 J/m². We collected cells immediately after treatment and used as substrate for a comet assay. On the other hand, LNCaP cells were pre-treated with 30 nM SS and 10 µM SM for 72 h. Total protein cell extracts were prepared, and substrates were incubated for 30 min at 37°C to analyze dimer excision capacity on damaged genomic DNA. The excision capacity (%) of the extracts was normalized to the T4 Endo V enzyme value that was considerd the 100% of excision capacity. Three biological replicates were tested in triplicate in three independent experiments. The mean tail intensity of each cell extract (N=3) was calculated and then a Student's t-test was performed.

4.2 Oligonucleotide-based biochip

A complementary method to analyse the excision capacity of protein extracts is the oligonucleotide (ODN) biochip (Figure 64). We used this *in vitro* miniaturized assay on biochip to simultaneously quantify several glycosylases activities contained in a nuclear cell extracts. ODNs contain different base lesions (each color point represents a lesion) and are labeled with a Cy3 fluorophore. They are then hybridized to complementary ODN addressed at specific locations on a glass support. Recognition and cleavage of the different lesions by the different DNA-glycosylases is quantified by measurement of fluorescence loss at each specific site.

The lesions spotted on the slide are 8oxoG paired with cytosine, adenine paired with 8oxoG (A-8oxoG), thymine glycol (Tg) paired with adenine (Tg-A), hypoxanthine paired with thymine (Hx-T), dihydrothymidine paired with adenine (dHT-A), ethenoadenine paired with thymine (EthA-T), uracil paired with guanine, and THF-A that is an analogue of abasic site, paired with adenine.

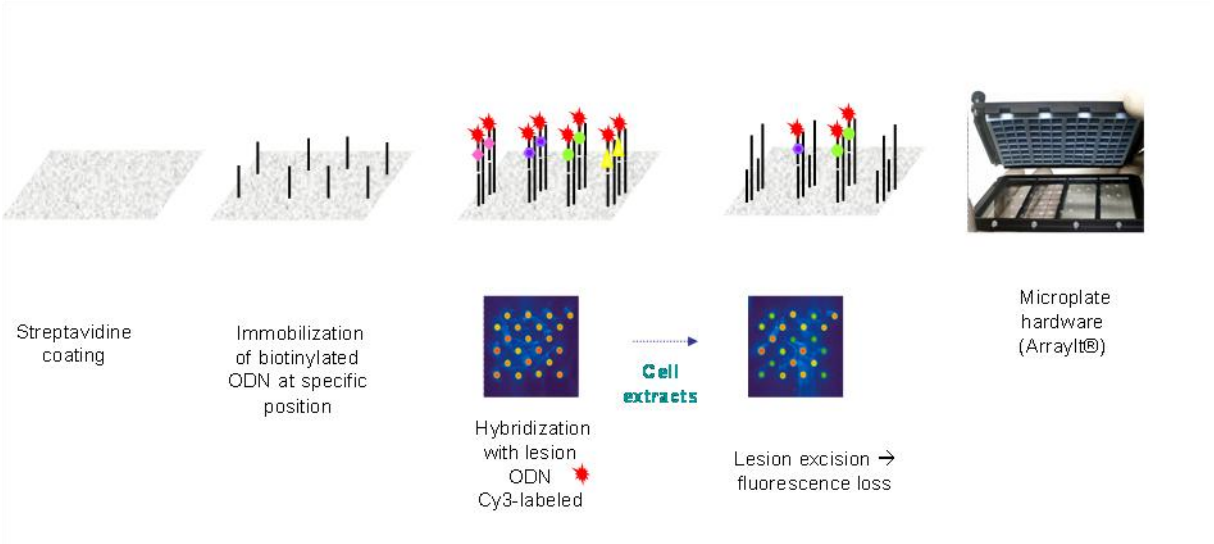

Figure 64: Summary of fundamental steps of ODN biochip assay. Several ODNs containing specific base lesions were immobilized together on the same support via sandwich-type hybridization (streptavidine-biotine). After cell extract incubation, hence excision activities of several damages could be monitored simultaneously

Figure 65 represents the ratio between the excision of ODN by the extracts from UVA irradiated cells and controls. The 8oxoG-C excision capacity was enhanced 10.9 fold in the SS extract following UVA irradiation, while it was increased only 2.06 and 2.42 fold in the NT and SM extracts following UVA irradiation. A-8oxoG corresponding to MYH recognition was excised 6.94 fold more for the SS condition, while it was increased 2.29 and 2.22 for NT and SM. The Tg-A excision capacity was increased 2 and 3 fold in the SS and SM following UVA stress. In contrast, Tg-A excision capacity for NT extracts was not really modulated by the irradiation. The Hx-T excision capacity was enhanced 7.8 and 3 fold in the SS and SM conditions following UVA irradiation, respectively, while it was unchanged in NT cell. dHT-A repair was slightly decreased for NT and SS cell extracts and it was increased 2 fold for SM cell. EthA-T excision capacity was increased by a factor 2 for SM and unchanged for NT

and SS cells, following UVA stress. The U-G and THF-A excision capacities were not modulated following UVA stress in any condition.

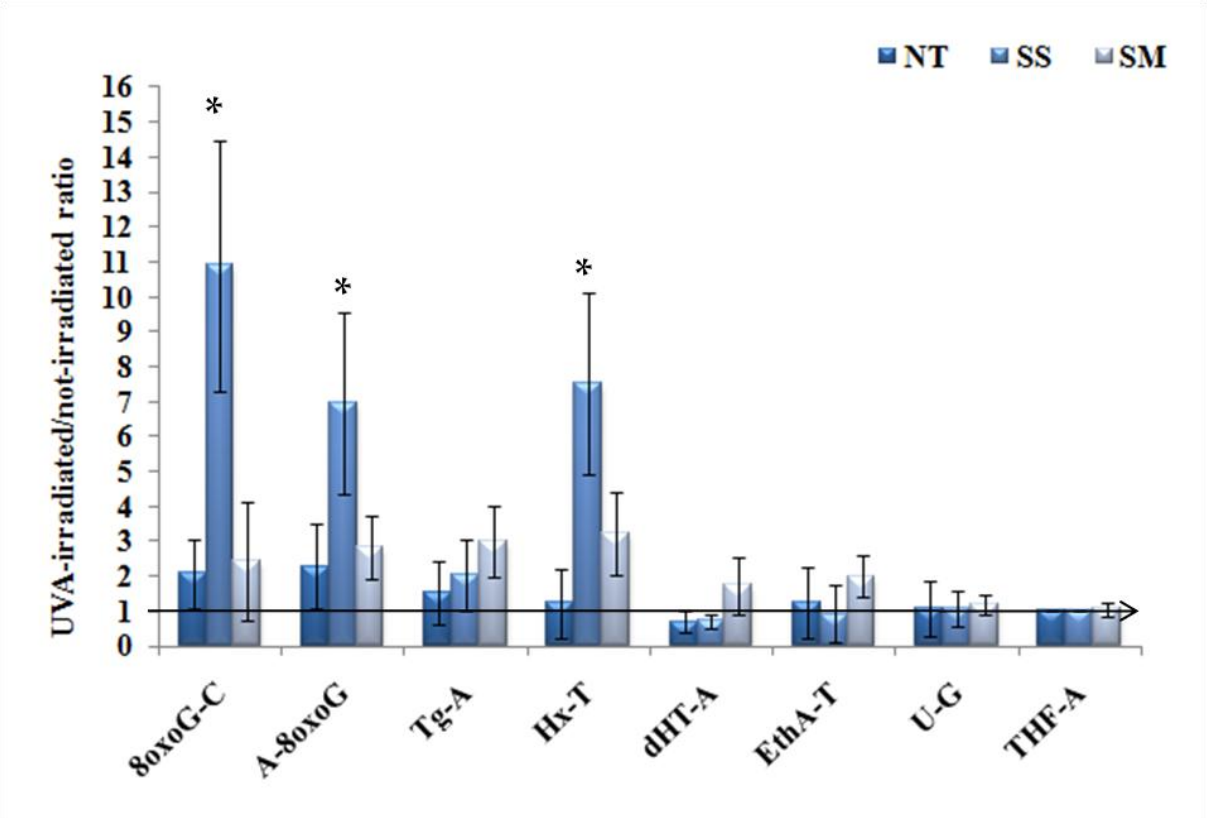

Figure 65: UVA-induced several excision activity of several modified DNA bases in Se-treated conditions. * p < 0.05 vs NT. LNCaP cell lines were cultured with or without SS or SM for 72 h and then were irradiated with UVA at 10 J/cm². Nuclear proteins (20 µg/mL) were then extracted and tested for their excision capacity on the ODN biochip for 30 min. The mean percentages of excision capacity were generated and the mean ratios of treated/non-treated were calculated for each lesion Three biological replicates were tested in triplicate in three independent experiments.

SS extract following MMS stress enhanced the excision of 8oxoG-C, A-8oxoG, Tg-A, and Hx-T of 5.3, 6.7, 1.8, 2.3 and 10 fold, respectively. Moreover, NT extract following MMS stress increased 8oxoG-C and A-8oxoG of 4.8 and 2.4 fold, respectively. Like UVA-irradiated samples the excision repair capacity of U-G and THF-A was unchanged for all conditions.

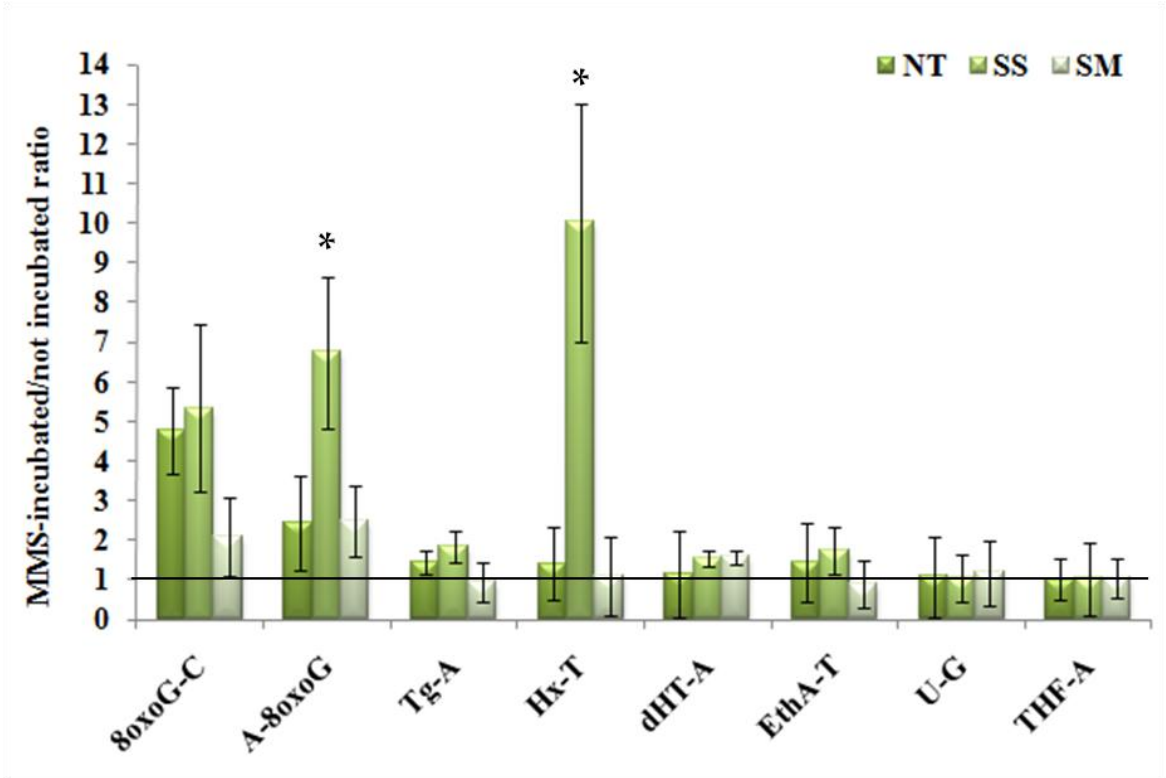

Figure 66: MMS-induced excision activity of several modified DNA bases. * p < 0.05 vs NT. LNCaP cell line was cultured with or without SS or SM for 72 h and then were incubated with 500 µM MMS for 15 min at 37°C. Nuclear proteins (20 µg/mL) were then extracted and tested for their excision capacity on the ODN biochip for 30 min. The mean percentages of excision capacity were generated and the mean ratios of treated/non-treated were calculated for each lesion. Three biological replicates were tested in triplicate in three independent experiments.

5. Discussion

This chapter was devoted to the investigation of the modulation of DNA repair by the base excision pathway in cells pre-treated or not with SS and SM and subjected to several stresses. We used different and complementary assays.

Comet assay study explored the repair rate of damaged DNA. The repair kinetics of DNA strand breaks had the same profile for NT, SS and SM cells following UVA irradiation. In the previous chapter, we found that, immediately after a UVA stress, SS and SM cells significantly decreased DNA stand breaks. The DNA strand breaks measured include SSBs, DSBs and AP-site that under alkaline condition are converted into strand breaks. These findings establish that pre-treatment with selenocompounds decrease DNA strand breaks formation but the repair efficiency was unchanged.

On the contrary, the repair kinetics of oxidative purines was faster 4 h after UVA irradiation for SS and SM conditions. Therefore, we concluded that SS and SM pre-treatment stimulate the repair of 8-oxoGua 4 h after the irradiation. The DNA repair system involved into repair of 8-oxoGua is BER. The glycosylases involved in the detection and excision of these lesions is OGG1.

An important question is why Se-stimulated 8-oxoGua repair in LNCaP cells? We hypothesized: (i) that selenium could act by increasing transcription and /or translation of hOGG1; this involves a larger amount of the glycosylase and thus a faster DNA repair; (ii) selenium increase the activity and the excision capacity of hOGG1, due to the antioxidant properties of selenoproteins, which could rebalance cell redox state after UVA radiation.

These findings led us to analyse the gene and protein expression to see if some BER-associated enzymes were modulated by selenium pre-treatment.

The qPCR analysis showed that hOGG1 was over-expressed at the basal level for the SS condition compared to NT. Yet, UVA irradiation led to a decrease in the expression of several DNA repair genes. Unlike qPCR, western blot analysis showed a slight increase in protein expression level of hOGG1 after selenium pre-treatment, but also after UVA radiation.

In order to test the second hypothesis, we used two complementary assays to evaluate the *in vitro* excision capacity of protein extract. The main differences of these two assays are the substrate nature and the proteic extracts. Comet assay was carried out on a damaged genomic substrate incubated with total protein cell extracts pre-treatment with SS or SM in absence of and additional stress. On the other hand, ODN biochip assay was carried out on a specifically damaged ODN incubated with nuclear protein extracts pre-treated with SS or SM but also stressed with UVA or MMS. These two approaches allow to asses on one hand the selenium influence on repair capacity at the basal level and on the other one whether selenium could modulate protein translocation after stress.

We examined the effect of Se on DNA repair capacity using a modified-Comet assay to measure excision activity of the protein extracts on damaged genomic substrate. Extracts of cells pre-treated with Se recognized and removed greater amounts of oxidized DNA bases than the NT extracts. Moreover, a clear bias toward improved response afforded by Se was observed for oxidative stress. In contrast, the repair capacity of cell extracts (NT, SS, SM) on MMS-incubated and UVC-irradiated substrates were similar using the Comet assay-based approach.

The pathways involved in the repair of lesions produced by the three stresses were quite different. Since UVC-induced photoproducts are repaired by the NER pathway, our results indicated that the pathways involved in NER were probably not affected by Se supplementation. However, selenium in the form of selenomethionine was shown to enhance NER as measured by the host-cell reactivation in human fibroblasts (Seo et al., 2002b). The different finding is probably to the different cell model used and also the time of pre-treatment (15 h).
The different repair response from Se pre-treated extracts to repair UVA/Riboflavin and MMS treated substrates was likely due to effects on the different glycosylases involved in the detection and removal of the two types of DNA damage. The glycosylase involved in the first step of 8-oxoGua removal is hOGG1, while that which detects methylated purines is N-methylpurine-DNA glycosylase (MPG). However, the predominant MMS-induced lesion is N^7MeG, which can be more mutagenic by conversion of the primary lesion to Fapy-N^7MeG, an adduct removable by OGG1 (Shrivastav et al., 2010). The variety of DNA damage caused by MMS treatment is likely to explain the higher excision activity of the cell extracts compared to that recognized by with Fpg or Endo III as extracts can support the excision of more kinds of lesions induced by MMS above that which can be recognized by the two individual enzymes.

In addition, a very interesting result was the ODN biochip analysis because it provides a global idea of the excision capacity by repair enzyme after a stress compared to the basal level. The ODN biochip allows us to detect any modulation of

the specific glycosylase excision capacities. In particular, we observed an increase of the activity of several specific repair enzymes after irradiation with UVA and mainly influenced by pre-treatment with selenium. The excision of 8-oxoGua was significantly higher only in cells pretreated with SS, suggesting large hOGG1 activity. Furthermore, this analysis allowed us to observe the increase in the excision of mismatched A-8oxoGua induced by MYH glycosylase.

This shows that the pre-treatment with SS not only reduces the formation of 8-oxoGua, but also increases the expression and efficiency of hOGG1 and MYH, essential glycosylases involve in the repair of this lesion. hOGG1 expression is just slightly increased by selenium supplementation but the excision capacity of these glycosylase is significantly increased by pre-treatment with SS. In conclusion, we believe that the action of a non-toxic amount of SS is mediated not only by the antioxidant properties of selenoproteins, but also by cell signaling involving BER repair system and in particular by modulation of protein status.

The improved repair of oxidized lesions obtained for pre-treated cells with SS and SM could be linked to the redox properties of enzymes whose activities are increased by Se supplementation. The AP site is the major product of OGG1 and its lyase activity is the rate-limiting step. Human APE1 significantly stimulates OGG1 activity in vitro (Hill et al., 2001; Sidorenko et al., 2007). Moreover, hOGG1 possesses critical redox-sensitive residues and being in the reduced state is important for 8-oxoGua DNA glycosylase activity. In particular, molecular epidemiologic case-control studies indicated that a polymorphic variant of hOGG1 (serine to cysteine amino acid substitution at position 326) is associated with a higher risk of developing several types of cancer (Chen et al., 2003). The OGG1-Cys variant is particularly sensitive to the cellular redox status, for example, OGG1-Cys enzymatic activity decreases after a pro-oxidant treatment (Bravard et al., 2009). These findings confirm the central importance of oxidative stress in carcinogenesis since it not only induces DNA damage but can also reduce the cellular protection against such stress. The redox sensitivity of the OGG1 pathway may explain how Se supplementation and its consequential stimulation antioxidant defences contribute to the maintenance of a

high hOGG1 activity explaining. Such a redox control also explains the enhanced excision activity achieved in Se-treated cell extracts for oxidative damage but not for agents whose exposure results in DNA alkylation or photoproducts.

Moreover, several studies showed that post-translational modifications of several DNA glycosylase, including OGG1, could modulate various aspects of DNA damage response. Phosphorylation and acetylation have been detected for OGG1.
It is known that OGG1 excises 8-oxoG and other damaged base substrates from DNA by attacking the N-glycosidic bond with Lys249 as the active nucleophile to form a transient Schiff base. After removal of the base lesion, the bound enzyme carries out the lyase reaction via β-elimination to cleave the DNA strand at the damage site and generates 3′-phospho-α,β unsaturated aldheyde and 5′-phosphate termini (Radicella et al., 1997). Unlike other glycosylases, OGG1 remains strongly bound to the resulting AP site without carrying out β-elimination (Zharkov et al., 2000).
Bhakat at al. showed that OGG1 is acetylated by p300 both *in vivo* and *in vitro* and they identify Lys338 and Lys341 as the major acetyl acceptor sites. Acetylation of OGG1 increases its *in vitro* turnover in the presence of APE1 (Bhakat et al., 2006). Because the rate-limiting step in 8-oxoG excision is dissociation of OGG1 from the product AP site, it is reasonable to postulate that acetylation enhances OGG1's turnover by weakening its interaction with the AP product. Displacement of OGG1 from the bound AP site by APE1 is enhanced when OGG1 is acetylated and its affinity for the AP site is reduced. Thus, OGG1-mediated repair of 8-oxoG (and FapyG or other substrate base lesions) is regulated by acetylation of the enzyme, in response to exogenous oxidative stress and not by changing the protein level. Eventually, homeostasis is restored when acetylated OGG1 (AcOGG1) is deacetylated by HDAC.
Xiang et al. showed that selenite treatment of LNCaP cells inhibited HDAC activity without significantly altering the levels of HDAC proteins (Xiang et al., 2008). This could explain a better OGG1 activity in cells pre-treated with selenium. In particular, ODN biochip analysis showed a significantly increase of OGG1 excision capacity of 8-oxoG modification. If selenite inhibited the activity of HDAC, this would involve

an inhibition of deacetylation of OGG1 that consequently would increase of AcOGG1 and enhance repair efficiency.

Alternatively, other studies also indicated that OGG1 phosphorylation state may change its turnover rate, in a similar fashion to how APE1 increases OGG1 turnover by displacing it from the abasic site. Moreover, OGG1 serine/threonine phosphorylation by Cdk4 stimulates 8-oxoG incision. Thus, it is possible that upon DNA damage the Cyclin D1/Cdk4 complex is still active and available to phosphorylate OGG1 in order to up-regulate DNA repair.

Several studies showed a relationship between selenium intake and kinase activity. Both selenite and SM, at nutritional doses, can up-regulated the expression of numerous cyclin-dependent kinase cdk1, cdk2, cdk4, cyclin B and cyclin D2 mRNA. In addition, Se increases total cellular phosphorylated proteins. Thus, the up-regulation of cdk1, cdk2, cdk4, cyclin B and cyclin D2 led to the promotion of cell cycle progression, particularly G2/M transition and /or the reduction of apoptosis, *in vivo* and *in vitro* (Zeng, 2002).

Moreover, post-transcriptional modification could modulate the activity of other glycosylases. Actually, another report suggests that MYH glycosylase activity is enhanced by phosphorylation. Interestingly, dephosphorylation of native MYH reduces its glycosylase activity on A/8-oxoG mismatches (Gu and Lu, 2001). This observation suggests that substrate specificity of DNA repair enzymes may be altered by post-translational modifications. Moreover, Lu and co-workers showed that UDG dephosphorylation by the p53-regulated phosphatase PPM1D reduced UDG activity, suggesting that PPM1D may inhibit BER by dephosphorylating UDG to direct its inactivation after completion of repair (Lu et al., 2004).

More interestingly, APE1 activity is post-transcriptionally modulated via redox modification by thioredoxin. Thioredoxin through its dithiol-reducing activity, reduces APE1 through a direct association that results in a heterodimer complex. Functionally, the interaction of APE1 and Trx enhances sequence specific DNA binding of AP-1 and p53 mediated by APE1 (Hirota et al., 1997; Ueno et al., 1999). In

turn, the redox activity of Trx is maintained by the TrxR selenoprotein. In our study, we found a significantly increase in TrxR activity in selenium pre-treated cells. This finding suggests an increase of TrxR turnover that can also influence Trx activity. Trx has a critical role in regulating cellular redox status and it modulates APE1 redox activity. Therefore, the translocation of Trx, which is stimulated by the different Se compounds, indicates that cellular redox regulation entails a complex cascade of events (Evans et al., 2000).

Chapter 4 – Optimization of a DNA repair assay in cellulo: Host cell Reactivation

Host Cell Reactivation (HCR) is a transfection-based assay in which cells repair specific damage localized in exogenous DNA. The interest of this assay is the possibility to study the DNA repair system directly in cells.

This methodology may involve two different approaches. First cells can be transfected with an undamaged plasmid and then cells were stressed. Consequently plasmid is damaged, and after that plasmid repair will be measured. Second, plasmid is separately damaged, then cells are transfected with the damaged plasmid and finally plasmid repair is measured. In this work we chose to use the second approach because our aim was to evaluate the DNA repair capacity of LNCaP cells just pre-treated or not with SS or SM in the absence of an additional stress. Plasmid was damaged by irradiation or by chemical agents (1). Thus, the host cells (NT, SS and SM) were transected with a damaged plasmid containing a reporter gene which has been deactivated due to the damage (2). The ability of the cells to repair the damage in the plasmid after it is introduced into the cell allows the reporter gene to be reactivated leading it to produce its reporting product (3). Through measurement of the activity of the reporter enzyme, the amount of damaged plasmid that a cell can repair and express can be quantified (Figure 67).

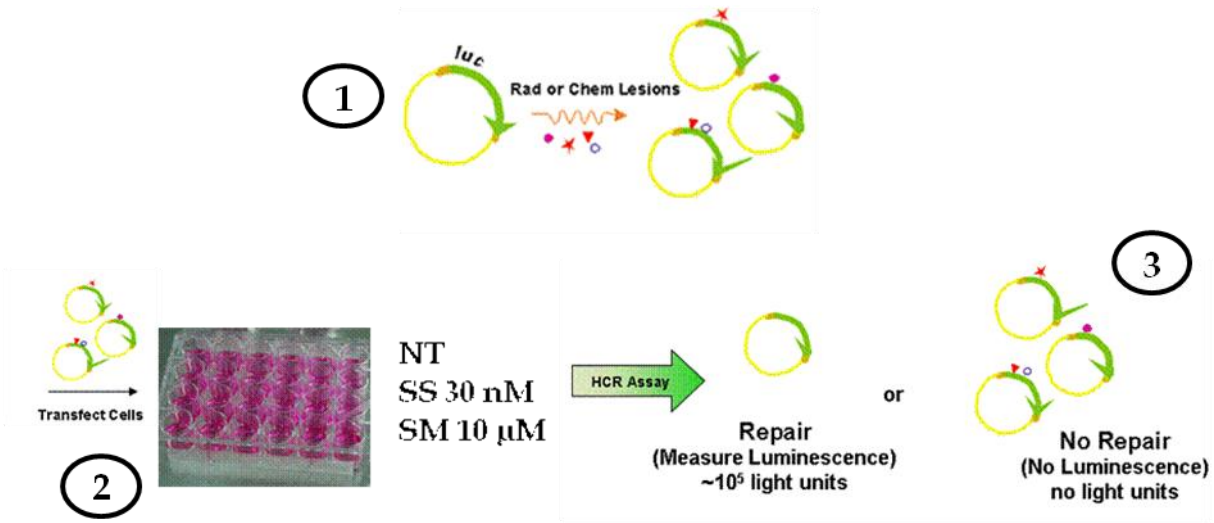

Figure 67: Schematically representation of the HCR assay

Because it involves repair of a transcriptionally active gene, when applied to specific damage the HCR assay measures the capacity of the host cells to perform both TCR and GGR NER; as well as all BER pathways.

The nature of the reporter gene, present in the plasmid sequence, can be varied and consequently allows to modify the type of measurement. The reporter gene can express β-galactosidase protein and plasmid repair capacity will be measured by a colorimetric assay (Pitsikas et al., 2005). Another frequently used reporter-gene expressed CAT (chloramphenicol acetyltransferase) and the detection is performed by a spectrophotometer (Athas et al., 1991). Alternatively, measurement of plasmid repair can be performed by fluorescence and for that its necessary that the plasmid contains a gene-reporter expressing for a fluorescent protein, such as GPF (Sattler et al., 2003). Finally, our choice fell on the plasmid with a reporter gene expressing a luminescent protein because the luminescence is more sensitive than UV visible and fluorescence.

This chapter describes the optimization of HCR assay using several damaged plasmids and transfection conditions. Different DNA repair pathways could be analyzed by this technique using different damaged plasmids.

Our previous results underlined the higher resistance of LNCaP cells pre-treated with selenocompounds after oxidative stress. Moreover, we observed a higher repair

capacity of 8-oxoGua. Until now, there is no HCR assay with a plasmid bearing 8-oxoGua as damage. So, in the last part of this work we tried to optimize an HCR assay adapted to our experimental conditions. Moreover, we carried out several kinetic analysis to evaluate the *in cellulo* repair capacity of LNCaP cells pre-treated or not with selenocompounds.

1. Co-transfection of pGLUC and pEGPF vectors

Our first approach to this assay involved the use of the Gaussia Luciferase (pGLuc-CMV) as gene reporter. The pGLuc-CMV plasmid contained the "humanized" coding sequence for the secreted Gaussia Luciferase under the control of the Cytomegalovirus (CMV) promoter, for constitutive expression in mammalian cells. This luciferase does not require ATP, and catalyzes the oxidation of the substrate coelenterazine in a reaction that emits light (470 nm). Figure 68 represents the DNA sequence of pGLuc with the position of the promoter, the luciferase gene sequence and the antiobiotic resistance sequences.

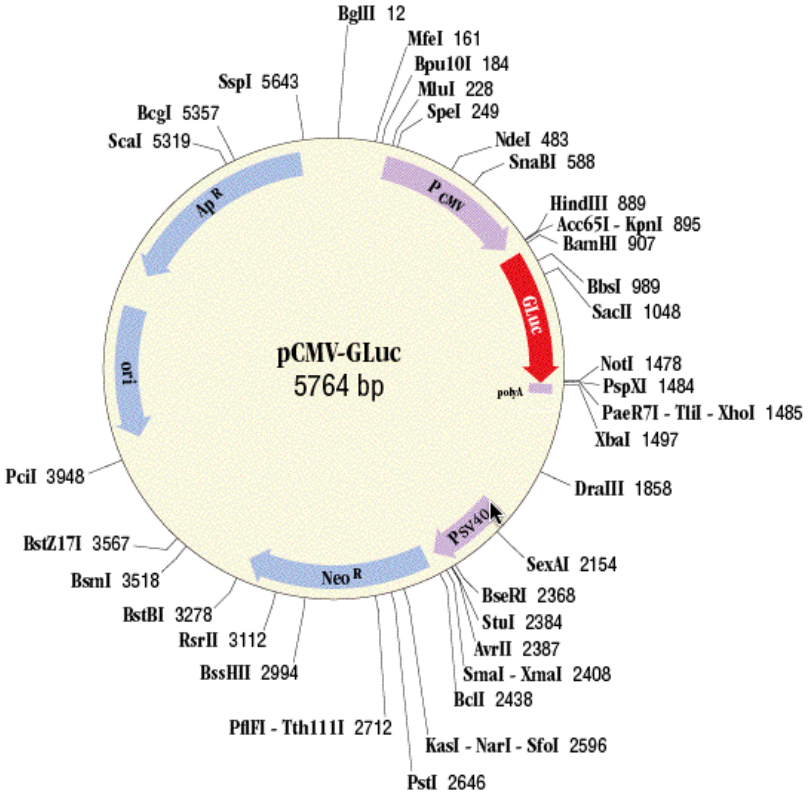

Figure 68: plasmid map of pGLuc

pGLuc-CMV was used as a experimental reporter in transfection assay in combination with other reporter vectors. It was co-transfected with a pEGFP-N3 vector as an undamaged control. pEGFP-N3 encodes a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488 nm; emission maximum = 507 nm.). Figure 69 represents the map of this plasmid that has a strong promoter (CMV).

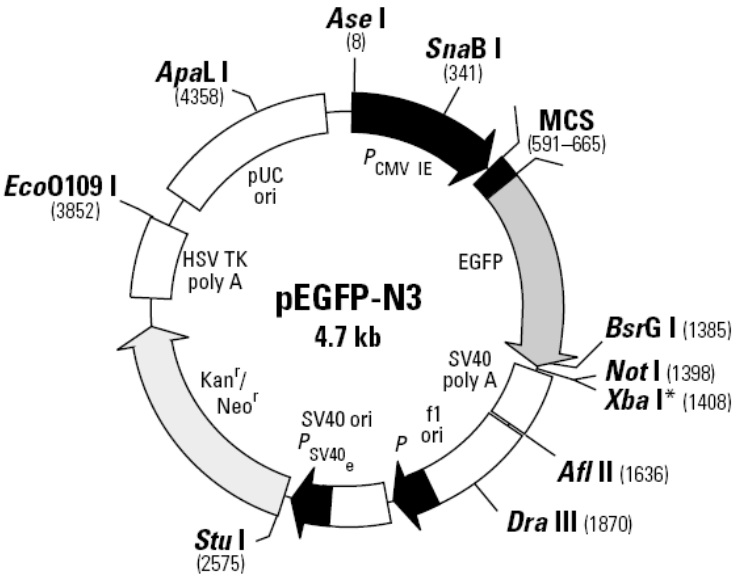

Figure 69: plasmid map of pEGFP-N3

1.1 Plasmid production

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. The purpose of this technique is to introduce a foreign plasmid into bacteria and to use bacteria to amplify the plasmid in order to make large quantities of it. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA (Figure 70). Then, cells were plated on antibiotic containing media.

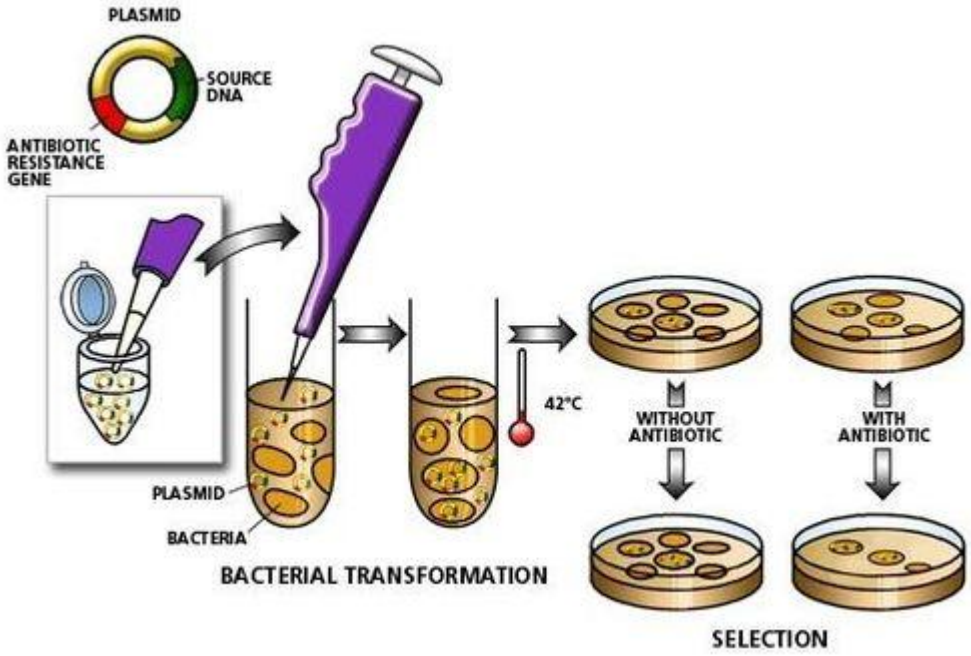

Figure 70: Schematic representation of bacterial transformation

We picked one competent colony and we amplified it in an Erlenmeyer flask at 37°C overnight and then we purified the plasmids by following the maxi-prep protocol.

To verify the quality of the plasmids, we carried out a native agarose gel electrophoresis analysis. Figure 71 shows the super-coiled form of the two plasmids, which confirm a good purification step.

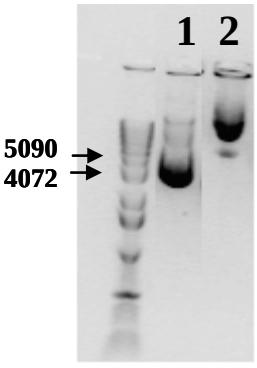

Figure 71: Representative picture of agarose gel of pGLuc and pEGFP. Line 1 pEGFP; line 2 PGLuc. 500 ng of plasmids was charged on a 1% agarose gel in TBE 0.5 % buffer.

1.2 Transfection

The next step was to transfect the plasmids into LNCap cells.

The cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h. Then cells were collected and re-plated (all conditions NT, SS and SM) for 24 h before transfection, by a specific transfection agent (Lipofectamine LTX).

Co-transfection of gene reporter with pEGFP was used to assess the transfection efficiency. Twenty-four hours after transfection we observed the 80% of transfection of pEGFP by a fluorescence microscope (Figure 72).

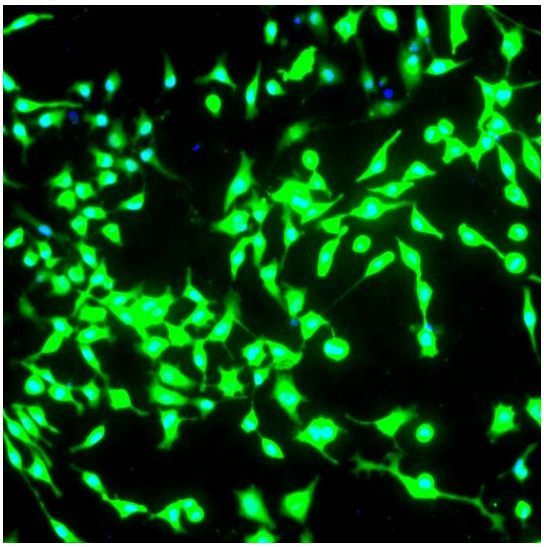

Figure 72: Fluorescence microscope picture of pEGFP-N3 24 h after transfection into LNCaP cell.

Thus, the transfection time was set at 24 h. Then, we also measured the luminescence of pGLuc 24 h after transfection. pGLuc possesses a natural secretory signal and upon expression is secreted into the cell medium. Therefore, lysis of the cells is not necessary. We measured the luminescence with the Gaussia Luciferase kit assay. We wanted to observe the signal stability and determine whether selenium could influence transfection of pGLuc, translation and secretion of the corresponding protein. The point at 24 h was the combination influence of the three previous parameters and we observed that there is not selenium influence (Table 4). The luminescence was expressed in Relative Light Unit (RLU).

	NT	SS	SM
RLU	$6{,}63*10^6 \pm 2{,}89*10^5$	$8{,}20*10^6 \pm 3{,}41*10^5$	$7{,}93*10^6 \pm 7{,}61*10^4$

Table 4: Luminescence of pGLuc in LNCaP (NT, SS and SM) cells 24 h after transfection.

Then we changed the culture medium and we measured the luminescence each hour to observe whether we reached a plateau. Figure 73 shows that 3 h after change of medium there is a weak luminescence signal. Moreover, the signal is the same for cells pre-treated or not with the two selenocompounds. So, we can affirm that selenocompounds did not also affect translation and secretion.

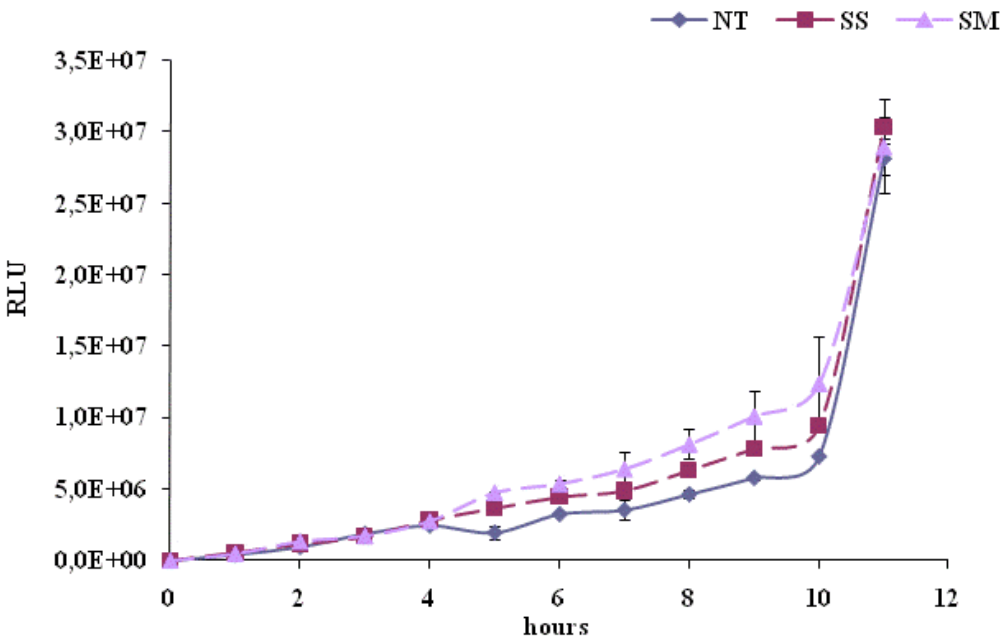

Figure 73: Kinetic study of pGLuc luminescence in LNCaP cell (NT,SS and SM). Cells were pre-treated with 30 nM SS or 10 µM SM, then collected and re-plated for 24 h. Then, NT, SS and SM cells were co-transfected with pGLuc and pEGFP for 24 h, then we changed media and we measured luminescence each hour.

The next step was to irradiate pGLUC with increasing dose of UVC radiation or UV/visible light in the presence of different concentrations of Riboflavin. Unfortunately, we found several problems principally linked to an inconstant luminescence activity of pGLUC. This problem is due to the absence of a control gene reporter that could minimize all the sources of variability (Table 5). This protocol was abandoned.

	NT	SS	SM
Non-irradiated pGLuc	4,38E+06 ± 1,73E+05	1,19E+07 ± 4,07E+05	1,80E+07 ± 2,30E+05
	7,54E+06 ±1,08E+06	9,88E+06 ± 3,75E+05	1,14E+07 ± 1,49E+06
	6,16E+06 ± 4,59E+04	5,39E+06 ± 1,15E+06	8,81E+06 ± 3,29E+05
UVC-irradiated pGLuC	6,20E+04 ± 1,22E+03	1,63E+05 ± 4,27E+03	1,77E+05 ± 5,41E+03
	9,62E+05 ± 1,87E+05	1,30E+06 ± 6,56E+04	1,45E+06 ± 2,87E+05
	5,87E+05 ±2,70E+04	2,52E+05 ±2,67E+04	8,32E+05 ±2,30E+04

Table 5: Luminescence of pGLuc (undamaged and irradiated with UVC) in LNCaP (NT, SS and SM) cells 24 h after transfection. Three biological independent experiments were carried out.

2. Co-transfection of Firefly and Renilla vectors

Dual reporters are commonly used to improve experimental accuracy. The term dual reporter refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the experimental reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected control reporter provides an internal control that serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency, can be also eliminated. Thus, dual-reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences.

We decided to use a co-transfection of other two plasmids; *Renilla* vector is used as a control to normalize the firefly. Firefly and *Renilla* luciferases, because of their

distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements. These differences make it possible to selectively discriminate between their respective bioluminescent reactions.

Firefly luciferase is a 61kDa monomeric protein that does not require post-translational processing for enzymatic activity (Wood et al., 1984). Thus, it functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2. Under conventional reaction conditions, the oxidation occurs through a luciferyl-AMP intermediate that turns over very slowly. As a result, this assay chemistry generates a flash of light that rapidly decays after the substrate and enzyme are mixed.

The plasmid used was the pGL4.13[*luc*2/SV40] with a strong SV40 promoter. The vector map is reported in Figure 74. This vector contains the SV40 early enhancer/promoter region, which provides strong, constitutive expression of luciferase in cells.

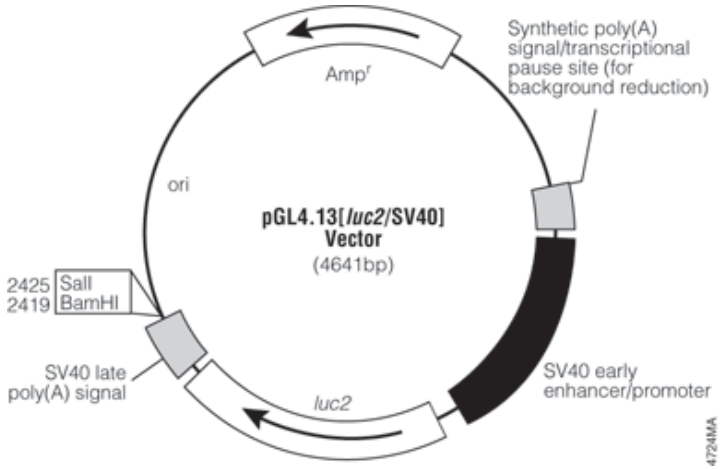

Figure 74: plasmid map of pGL4 vector

Renilla luciferase, a 36kDa monomeric protein, is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis* (Matthews et al., 1977). However, like firefly luciferase, post-translational modification is not required for its activity, and the enzyme may function as a genetic reporter immediately following translation. The luminescent reaction catalyzed by *Renilla* luciferase utilizes O_2 and

coelenterate-luciferin. The *Renilla* vector used is pRL-TK and the plasmid map is represented in Figure 75. The HSV-thymidine kinase promoter (pRL-TK) is relatively weak and may be particularly useful in providing constitutive expression of the Renilla luciferase control reporter.

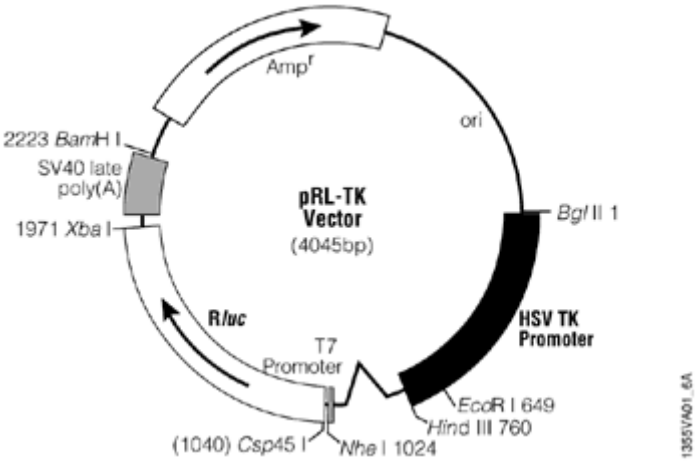

Figure 75: plasmid map of pRL-TK vector (Renilla)

2.1 Plasmid production and transfection optimization conditions

First of all we prepared the competent bacterial cells and we produced and purified the two plasmids as previously described (see chapter 4 section 1.1). We measured plasmid concentrations and we carried out a native agarose gel electrophoresis to analyze the plasmid quality. Figure 76 is a representative picture of the two purified plasmids and we observed that there are in the supercoiled form.

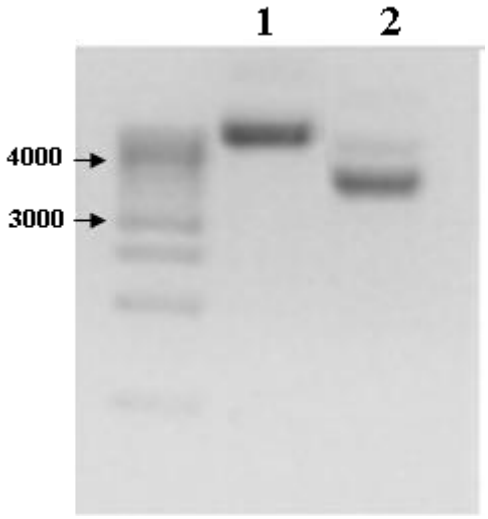

Figure 76: agarose gel of pGL4 and Renilla vectors. Line 1 pGL4; line 2 Ren. 250 ng of plasmids was charged on a 1% agarose gel in TBE 0,5 % buffer.

The next step was to determine the optimal ratio between conditions of pGL4 and *Renilla* for co-transfection. When the control or the experimental reporter vector, or both, contain very strong promoter/enhancer elements, the co-transfection can potentially affect reporter gene expression. This drawback is called *trans* effects between (Farr and Roman, 1992). Therefore, it is possible to add relatively small quantities of a control reporter vector to provide low-level, constitutive expression of that luciferase control activity.

We plated LNCaP cells for 24 h. Ratios of 1:1, 5:1, 10:1 and 50:1 with a constant total DNA amount for Firefly : *Renilla* combinations were co-transfected into LNCaP cells with Lipofectamine LTX. We measured the luminescence of pGL4 and Renilla 24 h after transfection with the Dual-Luciferase® Reporter Assay System (DLR).
In the DLR assay, the activities of firefly and *Renilla* luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement.
Figure 77 reports on the Y-axis the ratio between pGL4 and *Renilla* luminescence value. We can emphasize that the low value of 1:1 combination means a high Renilla RLU value. For a best performance the RLU value of control vector must be smaller. The ratio 10:1 presents a large standard deviation that connotes a random variability of pGL4 and Renilla luminescence. The ratio 50:1 is apparently a good compromise but the absolute value of Renilla luminescence is close to background noise. In conclusion the best ratio is 5:1. We have a small standard deviation that reflects a good reproducibility and the RLU *Renilla* values are ~300 fold lower than pGL4 but higher than background noise. The RLU values are reported in Table 6.

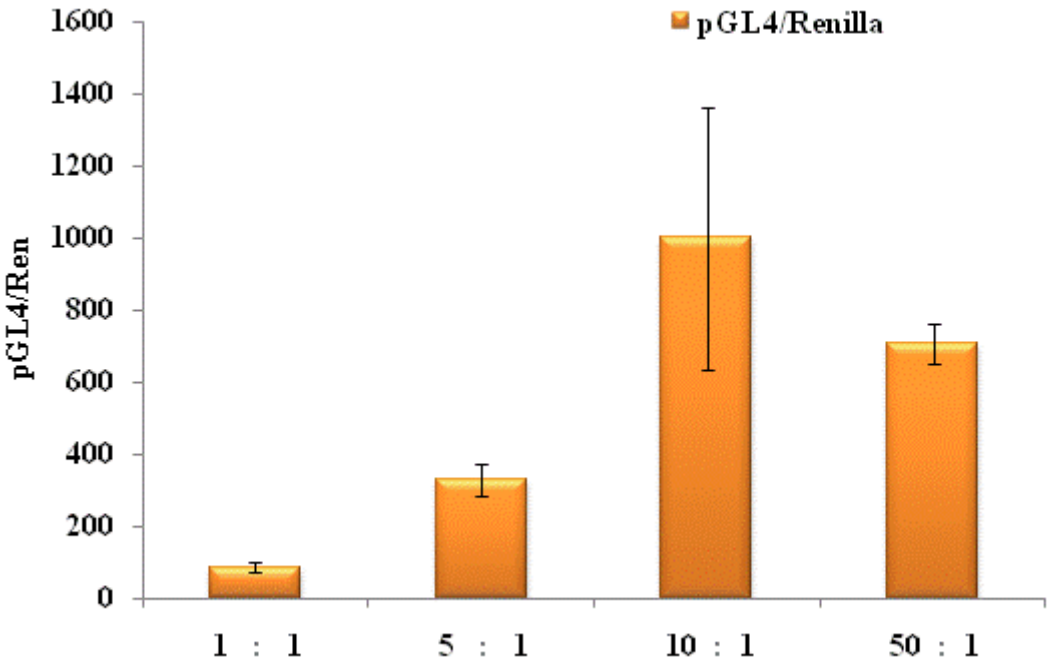

Figure 77: Evaluation of the better ratio pGL4 : Renilla to avoid *trans effect*. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h before transfection. Different amount ratio pGL4:Ren was used (1:1; 5:1; 10:1; 50:1). Luminescence was measured 24 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Two biological replicates were tested in triplicate in two indipendant experiments.

	1 : 1	5 : 1	10 : 1	50 : 1
pGL4	1,3E+06 ± 2,3E+05	1,7E+06 ± 2,0E+05	7,0E+05 ± 1,8E+04	3,0E+05 ± 9,6E+03
Renilla	1,5E+04 ± 1,1E+03	5,2E+03 ± 5,8E+02	3,6E+02 ± 6,0E+01	4,3E+02 ± 4,9E+01

Table 6: RLU values for pGL4 and Renilla 24 h after transfection

In all following experiments we will use a pGL4 : *Renilla* ratio of 5:1.

Afterwards we optimized the transfection step. We tested different amounts of total DNA and concentrations of Lipofectamine LTX.

We can observe, in the Figure 78, that the results for 500 and 750 ng of plasmid are similar, but for the following experiments we choose to use 500 ng (400 ng pGL4 and 100 ng Renilla) and a concentration of 2.5X of lipofectamine. The unit concentration represents the amount of lipofactamine compared to the amount of plasmid.

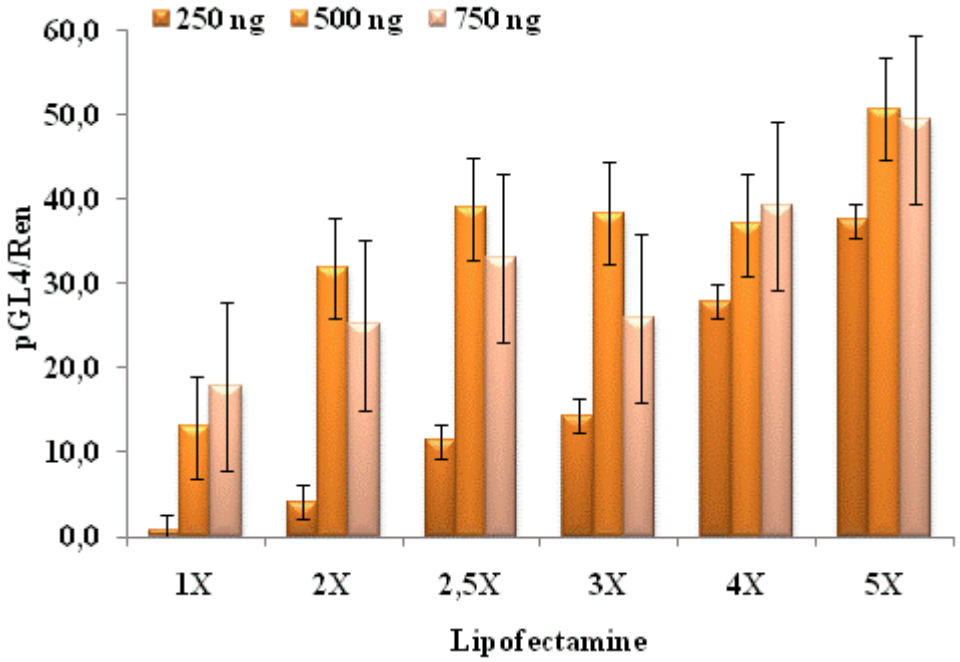

Figure 78: Optimization of transfection. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h before transfection. pGL4:Ren (5:1) was transfected.We used different amount of plasmid and different concentrations of Lipofectaine LTX for 24 h. Luminescence was measured 24 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Two biological replicates were tested in triplicate in two indipendant experiments.

Then, we pre-treated LNCaP cells with or without 30 nM SS or 10 µM SM for 72h, then we collected cells and we re-plated them for 24h. We co-transfected cells with pGL4 and Renilla in 5:1 ratio (500 ng total) using Lipofectamine LTX (2,5X) for 24 h. The luminescence of pGL4 was the same for all three conditions, as shown in Figure 79. This results means selenium pre-treatment did not affect the transfection and translation of two plasmids 24 h after transfection.

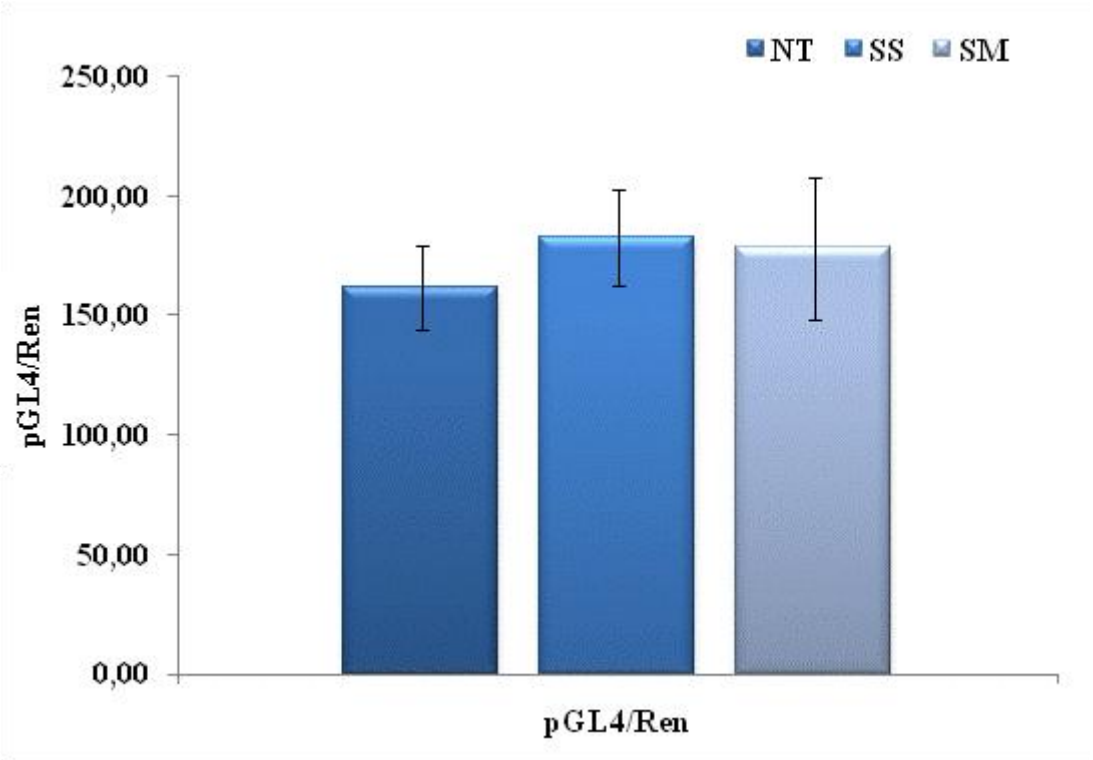

Figure 79: pGLuc/Renilla luminescence in LNCaP cell (NT,SS and SM) 24 h after transfection. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 24 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Three biological replicates were tested in triplicate in three indipendant experiments.

2.2 Riboflavin and UV visible light plasmid treatment

The aim of this assay was to evaluate the LNCaP repair capacities of a damaged plasmid transfected into cells.

We specifically damaged pGL4 by incubation with Riboflavin and UV/visible light to induce to formation of 8-oxoGua. We tested different concentration of Riboflavin in order to induce an increasing amount of 8-oxoGua. Moreover, we wanted to characterized the amount of 8-oxoGua formed. The oxidized base was revealed by incubation with Fpg glycosylase followed by gel electrophoresis. This assay allowed us to check the absence of strand breaks. A control sample was incubated only with a buffer under the same conditions (Figure 80). Because the plasmid is supercoiled one

or more breaks leads to less coiled form or to the circular form or linear form. It is possible that circular and linear form could overlap.

In the Figure 80 we can observe that the undamaged plasmid is mostly supercoiled when it is incubated either with buffer or Fpg.

After incubation with the buffer, the plasmid oxidized at different concentrations of riboflavin is still supercoiled. This result is very important because it suggests that the damaged plasmid were not cleaved and could be successfully transfected into cells. After incubation with Fpg, increasing amount of nicked form of the plasmid appears at growing dose of riboflavin. These findings confirm that upon irradiation we were able to specifically induce the formation of 8-oxoGua.

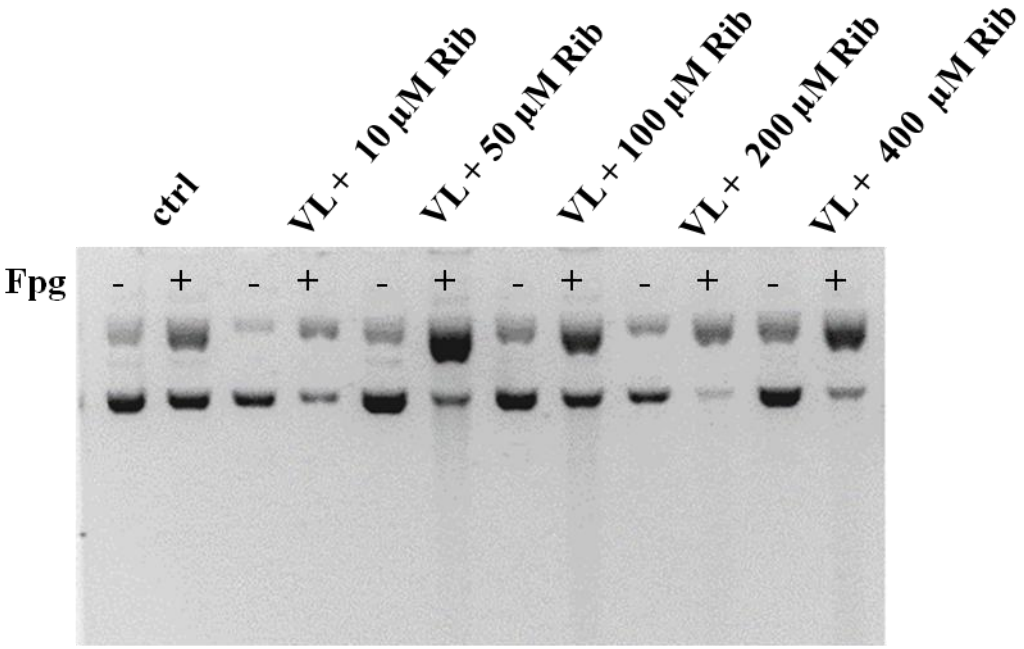

Figure 80: Agarose electrophoresis gel of pGL4. The plasmid was irradiated UV/visible light with a growing concentration of Riboflavin (10, 50, 100, 200 and 400 µM). The different samples were incubated with Fpg or only with a buffer for 30 min at 37°C.

In addition, for a quantitative analysis of 8-oxoGua amount we hydrolyzed pGL4 and we performed LC-MS/MS analysis. Figure 81 shows the amount of 8-oxodG for 10^6 bases. The plasmid measures 4.6 kb so we obtained almost 3, 4, 8 and 10 8-oxoGua/plasmid respectively to growing Riboflavin concentration.

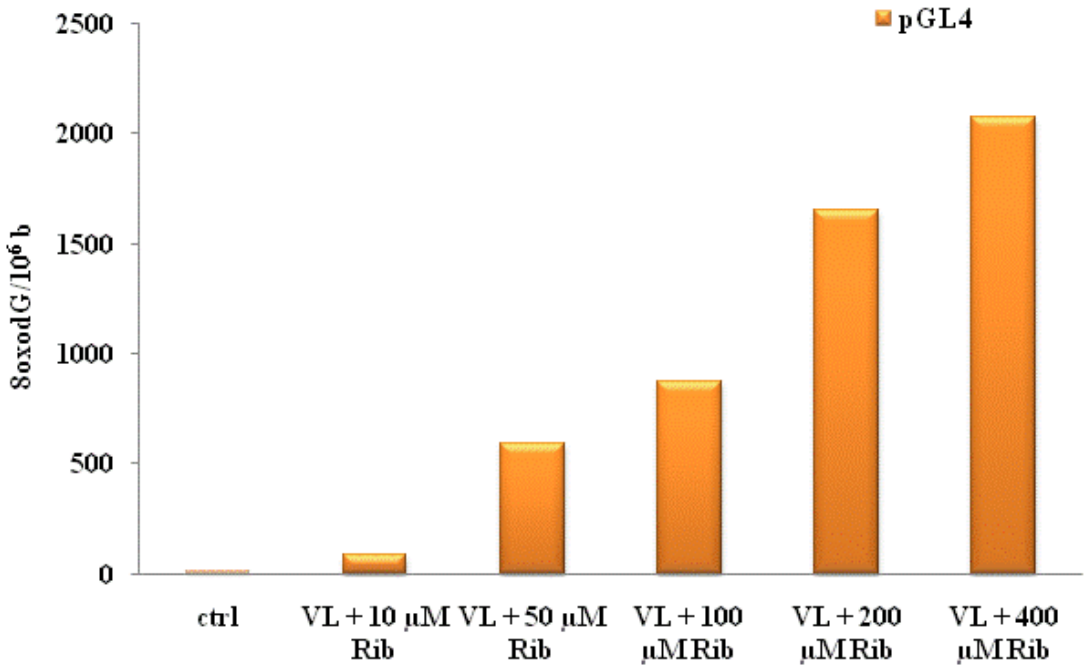

Figure 81: LC-MS/MS analysis of 8-oxodGua after pGL4 hydrolysis. pGL4 was irradiated with UV/visible light with increasing concentration of Riboflavin to induce 8-oxoGua formation, then it was hydrolyzed and quantified by LC-MS/MS.

2.2.1 Repair capacity of LNCaP cells pre-treated with SS or SM

We pre-treated LNCaP cells with 30 nM SS and 10 µM SM for 72 and then we collected cells and we re-plated in 24-well dishes for 24 h. Then, LNCaP cells were co-transfected (NT, SS and SM) with pGL4 and *Renilla* to observe the 8-oxoGua repair capacity 24 h after transfection. The damaged plasmid was less efficiently translated and the luminescence decreased when compared to undamaged pGL4 (Figure 81). The luminescence of damaged plasmid transfected in NT did not really change with the increase of damage. The plasmid transfected in SS and SM cells is apparently well repaired when compared to undamaged pGL4. Apparently plasmid transfected in SM cells underwent better 8-oxoGua repair independently from the Riboflavin concentration. Unfortunately, reproducibility was poor as shown by the large standard deviation.

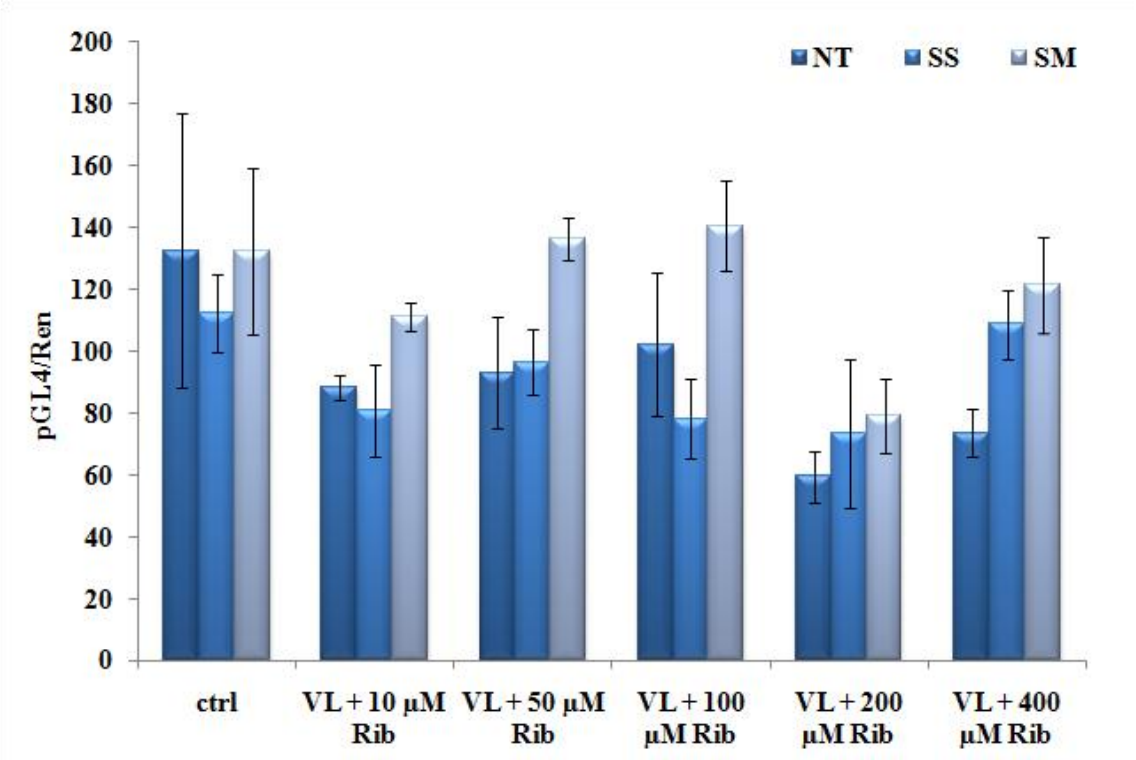

Figure 82: HCR assay of pGL4 irradiated with UV/visible light and several concentrations of Rib and then transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 24 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Three biological replicates were tested in triplicate in three independent experiments.

In order to optimize the assay, we performed a kinetic study. We prepared LNCaP cells as previously described and we co-transfected with pGL4 and Renilla. Either undamaged or damaged pGL4 were transfected into cells. We chose two concentrations of Riboflavin (10 and 400 µM) for the preparation of the last samples. We measured the luminescence at different times: 9, 24 and 48 h after transfection.

At first, we analyzed the kinetic response for the luminescence arising from undamaged pGL4. The point at 24 h confirms the result of the previous analysis where we did not observe any difference between three conditions (Figure 83). Yet, a different response was obtained at 9 h between the conditions. In NT condition pGL4 transfection and translation was apparently faster compared to SS and SM. Unexpectedly, an opposite trend was observed at 48 h. This time-course response was reproducible, although the absolute values of luminescence varied.

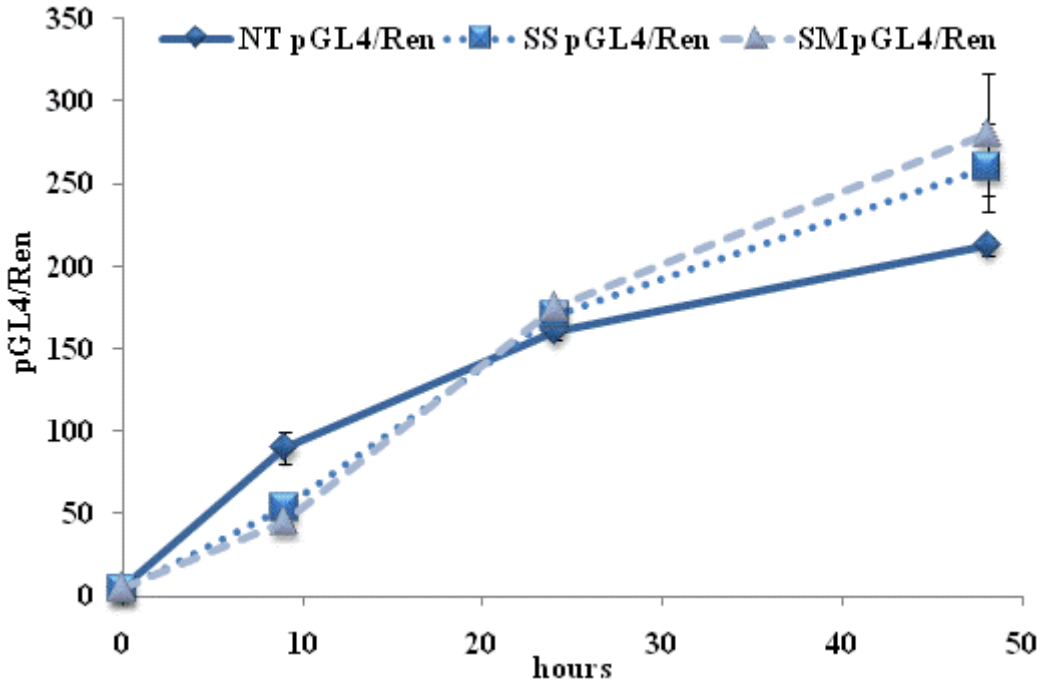

Figure 83: Kinetic HCR assay of pGL4 transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 9, 24 and 48 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Three biological replicates were tested in triplicate in three independent experiments.

The same response kinetic profile is observed for the repair of pGL4 10 µM Rib (Figure 84). At 24 h there are not differences between the luminescence ratios for all three conditions. On the contrary, 9 h after transfection the pGL4/Ren ratio was higher in NT cells than SS and in particularly compared to SM condition. The bias is completely inversed 48 h after transfection and in SM condition luminescence was higher than NT. The difference among the luminescence ratio of the three conditions (NT, SS and SM) could be affected by a different repair of 8-oxoGua.

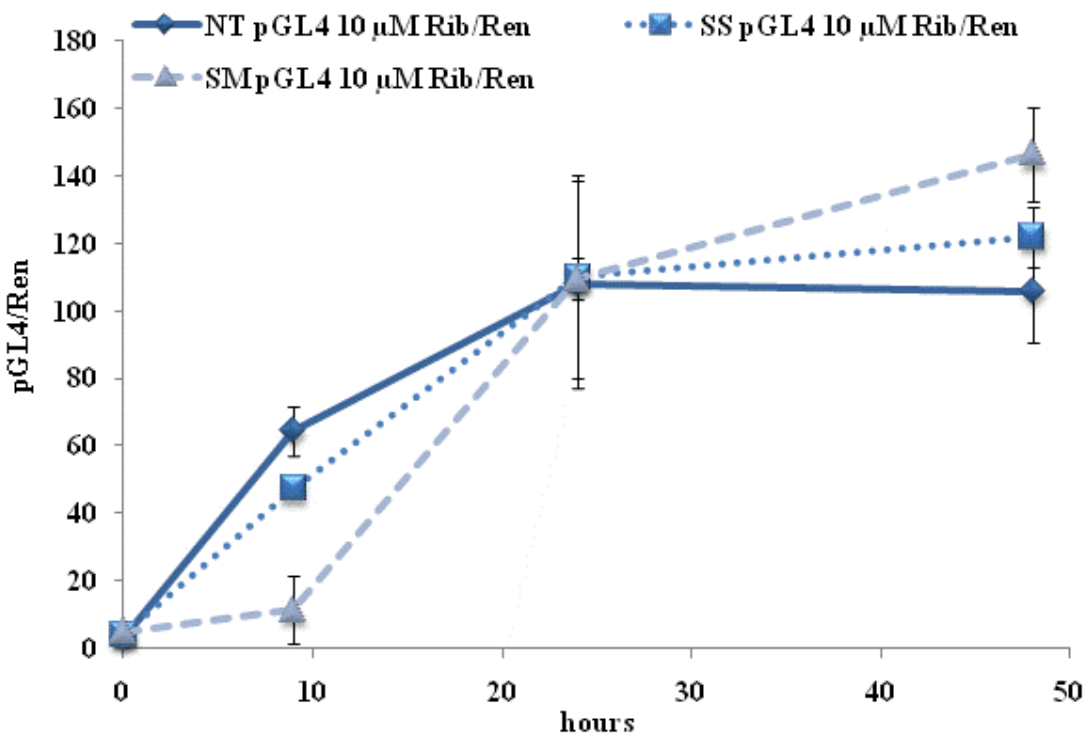

Figure 84: Kinetic HCR assay of pGL4 irradiated with UV visible light and 10 µM Rib and then transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 9, 24 and 48 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Three biological replicates were tested in triplicate in three independent experiments

The kinetic profile of the plasmid incubated with 400 µM of Riboflavin and irradiated with UV/visible light is similar (Figure 85). Transfection in NT cells favors to the translation of pGL4. Unlike the other kinetic profile, 24 h after transfection SS and SM cells express more pGL4 as compared to NT.

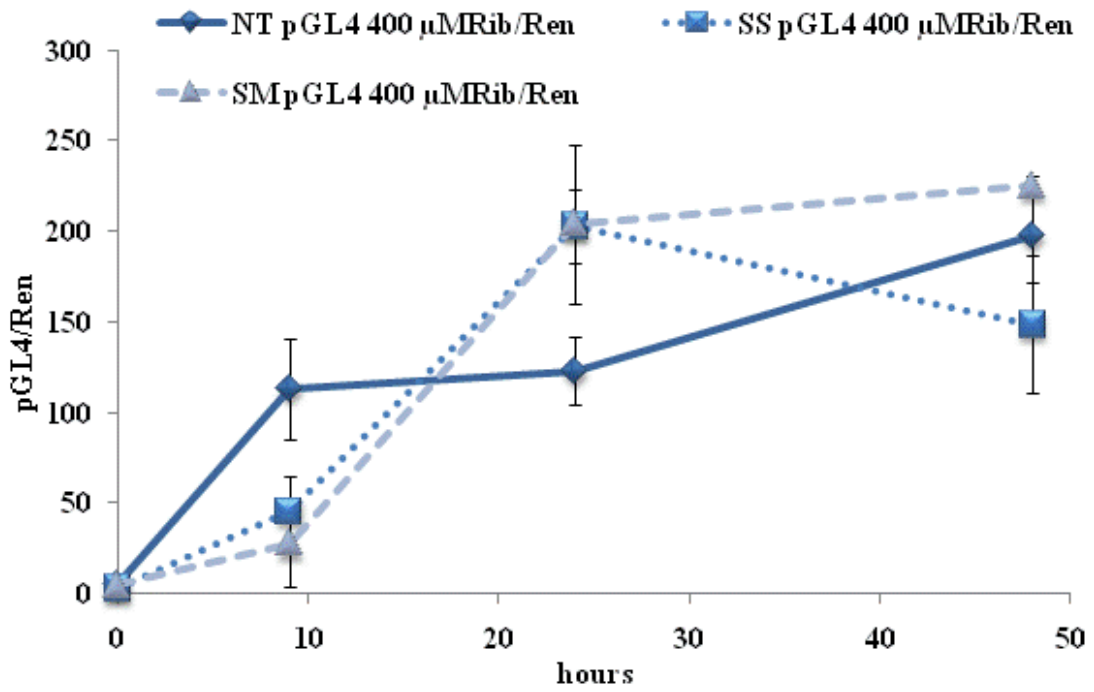

Figure 85: Kinetic HCR assay of pGL4 irradiated with UV visible light and 400 µM Rib and then transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 9, 24 and 48 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Three biological replicates were tested in triplicate in three independent experiments

Translation of 8-oxoGua containing plasmid was more complex than expected. It is clear that for all Riboflavin concentrations, 9 h after transfection the NT cells exhibited an optimal translation of pGL4. The point at 24 h showed no difference between NT, SS and SM except for pGL4 400 µM Rib where SS and SM condition seemed to repair faster damaged plasmid. Finally 48 h after transfection, SS and SM conditions led to a faster repair and pGL4 translation, except for pGL4 400 µM Rib where no differences were observed.

The fact that 8-oxoGua is not a blocking lesions could explain these different kinetic profiles dependent from the transfection time, damage amount and condition with or without selenium pre-treatment. Each kinetic time and each plasmid condition led to a different profile response between NT, SS and SM. At 9 h NT response is increasingly high compared to SS and SM with increasing of plasmid damage.

In contrast, at 24 h we did not observe any difference between NT, SS and SM for the three plasmid conditions. Finally, an inverse response compared to 9 h is observed 48 h after transfection. The difference in response between SM and NT, SS is increasingly high with the decreasing of plasmid damage. These findings suggest that the time is an important factor to determine the cellular behavior. Moreover, the different kinetic profiles, strongly affected by selenium pre-treatment and plasmid damage, also suggest that the results could be modulated at different steps, such as replication, transcription, repair and translation.

2.3 Dual-Glo® Luciferase Assay System

Because of the luciferase flash reaction (several seconds) hampers the realability of the measurement; we decided to use a last Luciferase kit that has the advantage to stabilize the luminescence signal.

The Dual-Glo® Luciferase Reagent (DGLR) can be added directly to cells in growth medium without washing or preconditioning. This reagent induces cell lysis and acts as a substrate for firefly luciferase, which has a half-life of approximately 2 hours instead of DLR. Addition of the Dual-Glo® Stop & Glo® Reagent quenches the luminescence from the firefly reaction and provides the substrate for *Renilla* luciferase in a reaction that can also be read within 2 hours (with a similar retention in signal).

The following experiments were carried out with this new detection kit.

2.4 UVC light plasmid treatment

The problem linked to the 8-oxoGua damage led us to irradiate pGL4 with a increasing dose of UVC to induce pyrimidine dimer formation. These lesions are blocking to translation. pGL4 was irradiated with increasing doses of UVC. To confirm the formation of dimers we hydrolyzed the pGL4 and we carried out an LC-MS/MS analysis to quantify the lesions amount.

Figure 86 shows the increasing formation of (6-4) PPs and CPDs.

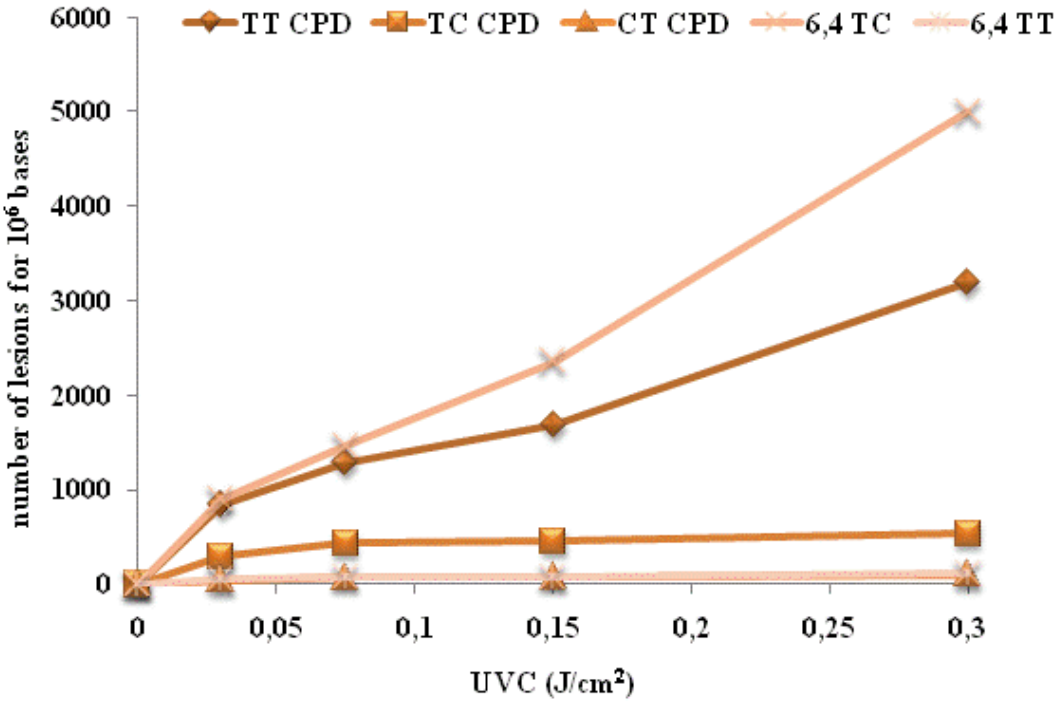

Figure 86: LC-MS/MS analysis of dimer after pGL4 irradiation with increasing UVC dose. pGL4 was irradiated with increasing doses of UVC to induce pyrimidine dimer formation, then it was hydrolysed and quantified by LC-MS/MS.

Once quantified the dimer amount, we co-transfected pGL4 and *Renilla* into LNCaP cells (NT, SS and SM) to observe the dimer repair capacity of cells 24 h after transfection (Figure 87). The plasmid was repaired between 15 to 90 % depending on the irradiation dose. We did not observe significant differences between NT, SS and SM cells except for the UVC dose at 0,15 J/cm² where SM cells apparently more efficiently removed pyrimidine dimers.

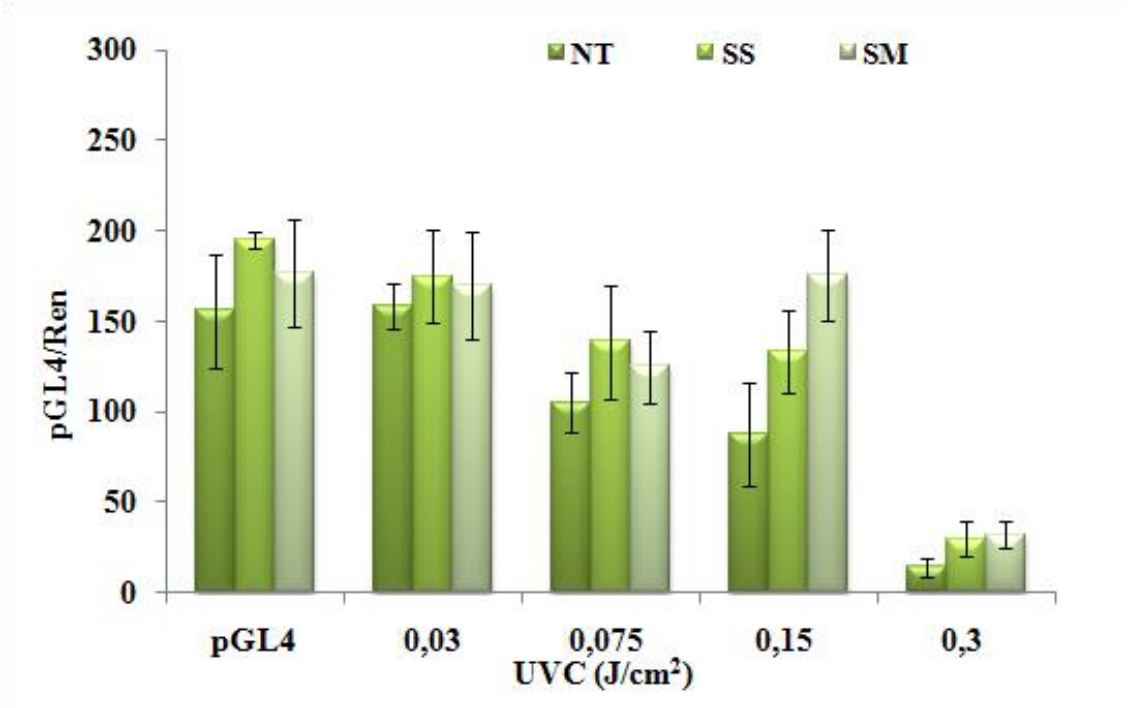

Figure 87: HCR assay of pGL4 irradiated with UVC increasing dose and then transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 24 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. Two biological replicates were tested in triplicate in two independent experiments.

As previously, we carried out a kinetic HCR assay and we choose only a UVC dose of 0,15 J/cm^2 because is the only one where we observed a different repair profile.

In parallel, we also realized the kinetic analysis of the luminescence signal of undamaged pGL4 (Figure 88). Unlike the kinetic profile measured with the DRL, and 8-oxoGua, the result with DGRL kit 9 h after transfection did not exhibit differences between the three conditions. On the contrary, this assay confirms a difference in pGL4 expression for SM condition compared to NT and SS 48 h after transfection.

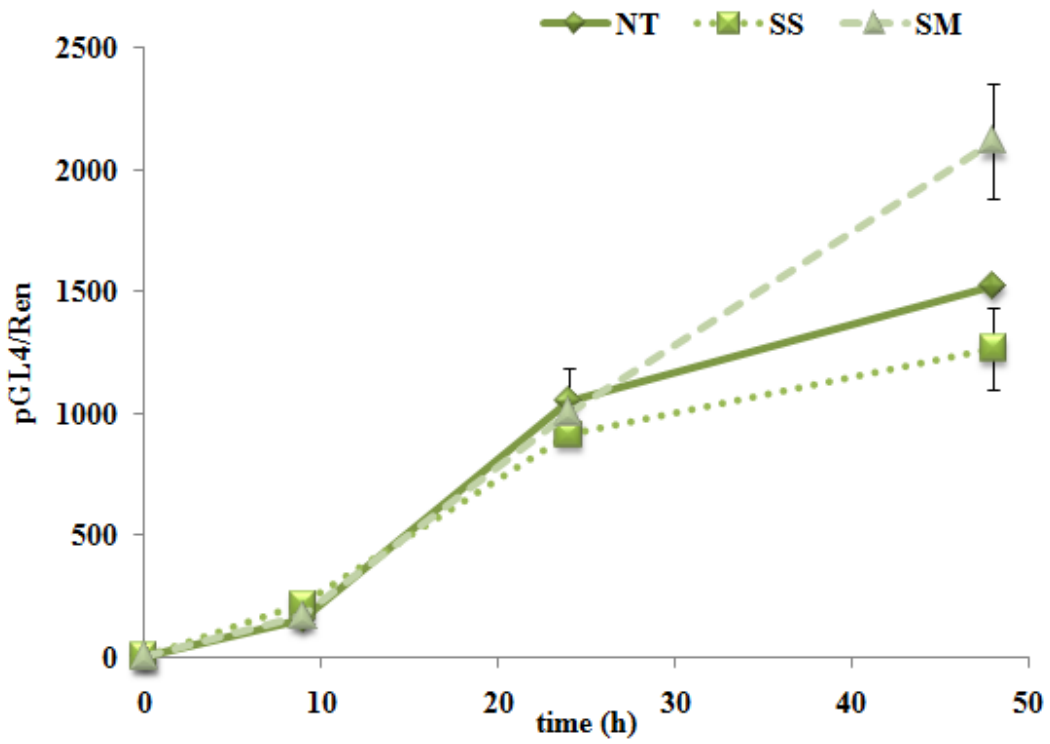

Figure 88: Kinetic HCR assay of pGL4 transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 9, 24 and 48 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. One biological replicates were tested in triplicate.

The kinetic profile of dimer repair was the same for all three conditions (Figure 89). These results support previous findings obtained in this work. Indeed, pretreatment with selenium in LNCaP cells did not produce any different cellular response after UVC irradiation. This means that the NER repair system is not affected by selenium. Instead, other studies made on fibroblast cells show a protective effect of selenium against UVC (Seo et al., 2002b). Seo at al. study used a different cell line model but more important their HCR protocol is quite different. Actually, they added SM 24 h after transfection and cells were incubated for 15 h with SM. On the contrary we pre-treated cells with selenium. Finally CAT activity was determined by Seo at al. 72 h after transfection; while us we measured luminescence over 48h after transfection.

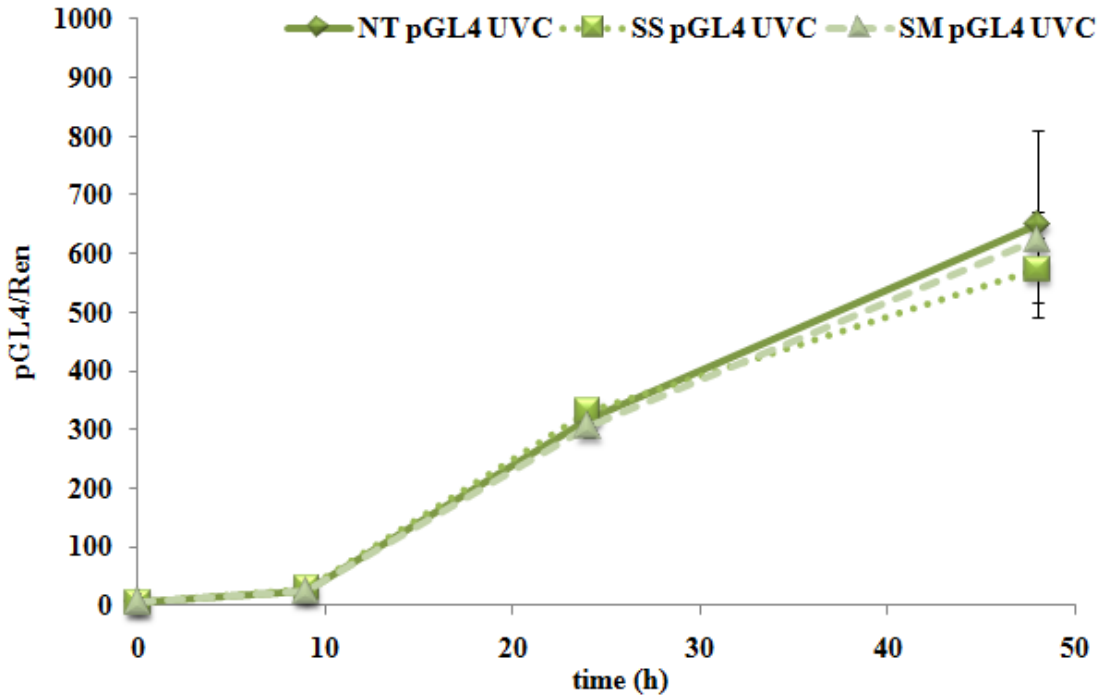

Figure 89: Kinetic HCR assay of pGL4 irradiated with UVC growing dose and then transfected in LNCaP cells. Cells were pre-treated or not with 30 nM SS or 10 µM SM for 72 h; then cell were collected and re-plated for 24 h. Moreover, NT, SS and SM cells were co-transfected with pGL4 and Renilla (5:1). Luminescence was measured 9, 24 and 48 h after transfection in LNCaP cells and the ratio between pGL4 and Renilla RLU values is reported on Y-axis. One biological replicates were tested in triplicate.

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Concerning HCR assay, we will performed future experiments using the new luminescence kit to have a clearer response about repair kinetics of 8-oxoG in the plasmid. Moreover, we could test Seo et al. protocol to compare the results in the same condition.

Furthermore, we could also imagine using another type oxidative damage on the plasmid that will be block the replication and the transcription, such as thymine glycol lesion.

The possibility to insert a specific modified base into a define position of the plasmid sequence could be a very interesting alternative compared to overall plasmid treatment. A main advantage would be to target essential elements such as the promoter region or the active site. Our laboratory is highly qualified in the synthesis of modified oligonucleotides.

Chapter 5 – General conclusions & Future perspectives

Selenium was discovered in 1817 and for several years it was described as toxic. In the 1950's, Schwarz's studies (1957) changed this idea forever. During the following years more and more studies were carried out about selenium supplementation and most of them found an inverse relationship between selenium intake and cancer risk incidence.

The anticarcinogenic actions of Se occur at the systemic, cellular and nuclear level. These actions may also involve the immune system and thus cannot be interpreted by a single mechanism. The anticarcinogenic action of Se also depends on its chemical form, dosage and the nature of the carcinogenic agent (Schrauzer, 2000).

Until now its mechanisms of action are not well understood. This work was carried out in order to better elucidate the possible cellular pathways which link selenium supplementation and genotoxicity.

In order to consolidate the bases of our work, we used a reliable cell model sensitive to selenium supplementation. It is known that several selenoproteins polymorphisms may cause an insensibility to selenium supplementation. The alteration of these selenoproteins could prevent the response of cellular models. Fortunately, even if they are prostate cancer cells, LNCaP cells are not classified as presenting selenoprotein mutations. Moreover, LNCaP cells are p53-wt, which suggests that apoptosis, cell cycle arrest and DNA repair are normally regulated.

For future analysis, it will be necessary to validate our results on primary cell lines, such as primary cell culture of prostate or other epithelial cells such as keratinocytes and fibroblast.

In our work, we showed the importance of the form and the micronutrient concentration to use. In addition, it should be emphasized that the protective effect of selenocompounds is observed at low concentrations, especially for SS. This

compound is often used at high concentrations for its pro-apoptotic properties; in contrast to its impressive pro-apoptotic effect, we have emphasized its preventive effect at low nanomolar concentrations.

Subsequently, another remarkable point of this work was the protection of selenium against exposure to cytotoxic agents, in particular oxidative type.
The most important selenoproteins, GPx and TrxR are universally known for their involvement in maintaining the redox balance of cells.
The importance of these two selenoproteins and the influence of selenium supplementation could be emphasized using GPx and TrxR knockout cells. This perspective can highlight whether protection against oxidative stress could maintain in the absence of these proteins.
This suggests that one of the possible pathways of selenium action is through the reduction of ROS. Since the production of ROS begins in the mitochondria, it would be very interesting to analyze the repair of mitochondrial DNA in LNCaP cells pre-treated or not with selenium and upon UVA irradiation.

In contrast to most studies, our interest was not limited to a simple antioxidant mechanism. In fact, we investigated the events that could occur at the genomic level. Our analysis is more refined, highlighting that the pre-treatment with selenium was able to reduce the formation of DNA strand breaks, but also to reduce the formation of oxidized purines, with particular interest in the 8-oxoGua. This lesion represents a major product of DNA oxidation.

Our curiosity then naturally moved to the mechanism involved in the repair of 8-oxoGua in order to determine whether selenium decrease of 8-oxoGua was simply due to a reduction of ROS or whether selenium could also contribute to the repair of this damage.
The repair kinetic profiles highlighted that the pre-treatment with selenium also led to a faster repair of 8-oxoGua but not for DNA strand breaks.

The inevitable continuation was to investigate minutely on the proteins directly involved in repair of 8-oxoGua, such as hOGG1. In concordance with our previously results, we observed a slight increase in the expression of hOGG1. Moreover, selenium pre-treatment significantly increased the efficiency of excision capacity of this glycosylase, which allows a better repair of 8-oxoGua. Our hypothesis are based on a modulation of its post-transcriptional modification of OGG1 (acetylation and phosphorylation) modulated by selenium. An additional possibility could a protection against oxidation.

The ability of selenium to modulate post-translational phosphorylation and acetylation highlights a new and important role of selenium to control cellular homeostasis and modulate DNA repair. For this reason, we could also determine the activity of deacetylase and phosphatase in order to understand whether selenium directly affects post-translational modifications.

Our work clearly showed the increase in excision efficiency of the glycosylases activity is not necessarily correlated with an increase of gene expression and/or protein levels. As future analysis, it will be interested to evaluate the post-translational modification of OGG1 in order to observe whether the selenium pre-treatment in LNCaP cells increases the acetylation and/or phosphorylation of this enzyme. This analysis will be carried out with two complementary assays. Firstly, western blot analysis could be perfomed with specific antibody sensible to post-translation modifications. Secondly, proteomic analysis combining two-dimensional electrophoresis coupled to mass spectrometry and liquid chromatography could lead us to compare the levels of protein expression but also to the different post-translational modifications.

Moreover, we could also imagine studies of cells knock-out for certain proteins affected by selenium supplementation in order to observe and understand the directly link between the selenium intake and DNA repair mechanisms. In particular, hOGG1 knockout cells could highlight whether the selenium mechanism directly modulate OGG1 capacity to repair 8-oxoGua.

For future studies, p53 status appears as the next parameter to study, especially in relationship with post-transcriptional modifications.

As previously mentioned, LNCaP cells are p53 wild type. Upon activation p53 triggers a wide range of cellular response depending on the type of cell and stress. These processes include cell cycle arrest, DNA-repair, apoptosis, differentiation, senescence, inhibition of angiogenesis and metastasis (Vogelstein et al., 2000).

Multiple post-translational modifications, involving more than 35 different amino acids, are located throughout the p53 protein, in particular in the N-termianl domain, and many of these modifications have been shown to be critical for p53 functions. These modifications, including phosphorylation, acetylation, ubiquitination, glycosylation and sumoylation, lead to p53 stabilization as well as its transcriptional activity. It is not clearly understood whether selenium intake could affect these modifications, but a recently study on LNCaP cells showed a decrease of nuclear p53 accumulation in cells pre-treated with SS and SM after oxidative stress (Erkekoglu et al., 2011).

Moreover, Seo et al. reported that p53 activity can be augmented by antioxidant mechanisms, thereby protecting cells from DNA damage. SM modulated p53 activity by causing redox regulation of key p53 cysteine residues (275/277). The resulting conformational shift leads to the induction of p53 DNA-binding activity and the stimulation of DNA repair (Fischer et al., 2007; Gudkov, 2002). The effect of selenomethionine on p53 is not due to a direct interaction with p53 nor is it due to DNA damage. It requires the cellular protein APE1 that was previously shown to physically interact with p53. Inactivation of this protein blocks p53 modification by the selenium-containing compound. A very important parameter to be highlighted is that the redox state of APE1, which modulates the reduction of p53 and controlled by the thioredoxin reductase. As is clear from our results a selenium supplementation results in an increase of TrxR that could modulate the redox turnover in which p53 is involved.

However, the most unusual property of the modified p53 is that p53 becomes capable of activating DNA-repair machinery without affecting cell growth. Hence,

p53 can contribute to genomic stability not only by eliminating damaged cells, but also through directly activating a DNA-repair system (Seo et al., 2002a).
To consolidate our hypothesis, a recent study shows that long-term selenium consumption not only affects selenoprotein enzyme activities, tissue Se concentrations, and global genomic DNA methylation but also increases exon-specific DNA methylation of the p53 gene in a Se-dose-dependent manner in rat liver and colon mucosa (Zeng et al., 2011).
The several post-translational modifications of p53, according to the studies discussed above, could be modulated by selenium intake. So, based on the same type of experiments proposed for OGG1 could highlight the different levels of post-translational modifications of p53.

In conclusion, selenium ability to modulate the activity and post-translational modifications of different proteins involves the modulation of several sub-pathways that may seem unrelated (inhibition of HDAC, modulation of methylation) but lead to an increase resistance of cells to stress. Thus, we could image that the mechanism of action of selenium is represented by a delicate balance between activation and repression of protein activity that induces conformational changes of these proteins more or less directly involved in DNA repair and progression of cell growth.
Another important aspect of this work was underlining the higher efficiency of sodium selenite at nutritional concentration compared to selenomethione. These findings make it possible to re-evaluate the role of sodium selenite in the prevention studies.

As part of a man chemopreventive study we could imagine a comparative study of the DNA repair capacity system in individuals with different plasma baseline selenium concentration before and after selenium supplementation. The efficiency of our methodologies allows carrying out a screening of different patients groups.

Chapter 6 - Materials and methods

1. Cell culture

LNCaP human prostatic cancer cell line (lymph-node-derived androgen-sensitive cell line, normal for cell-cycle-related tumor suppressor genes p53 and retinoblastoma (Rb), [Wild type]) was a gift from Prof. Alan Diamond, University of Illinois (IL,USA). Cells were cultured in RPMI 1640 medium with 10% FCS and 1% penicillin/streptomycin in culture flasks at 37°C in 5% CO_2.

For selenium treatment, the media was supplemented with either 30 nM SS (Sigma) or 10 µM SM (Sigma) for 72 h. A control without treatment (NT) was included in each experiment.

2. Reverse transcription (RT) and real-time quantitative PCR (qPCR) analysis

Cells were plated in 100 mm petri dishes with or without 30 nM SS or 10 µM SM for 72 h and then were irradiated with UVA at 10 J/cm^2. Cells were collected 4 h after irradiation and dry pellets (~4*10^6 cell) were frozen at -80°C. Total RNA was extracted from each sample using the GenElute mammalian total RNA miniprep kit (Sigma) following the protocol. RNA quality was assessed using gel electrophoresis of 1% (w/v) agarose in TBE buffer 0.5 %. Samples were run at 90V in TBE buffer (containing 10 µl ethidium bromide 2.5 µg/ml). RNA bands were visualized under UV light. Total RNA was considered intact if two sharp 28S and 18S rRNA bands were visualized.

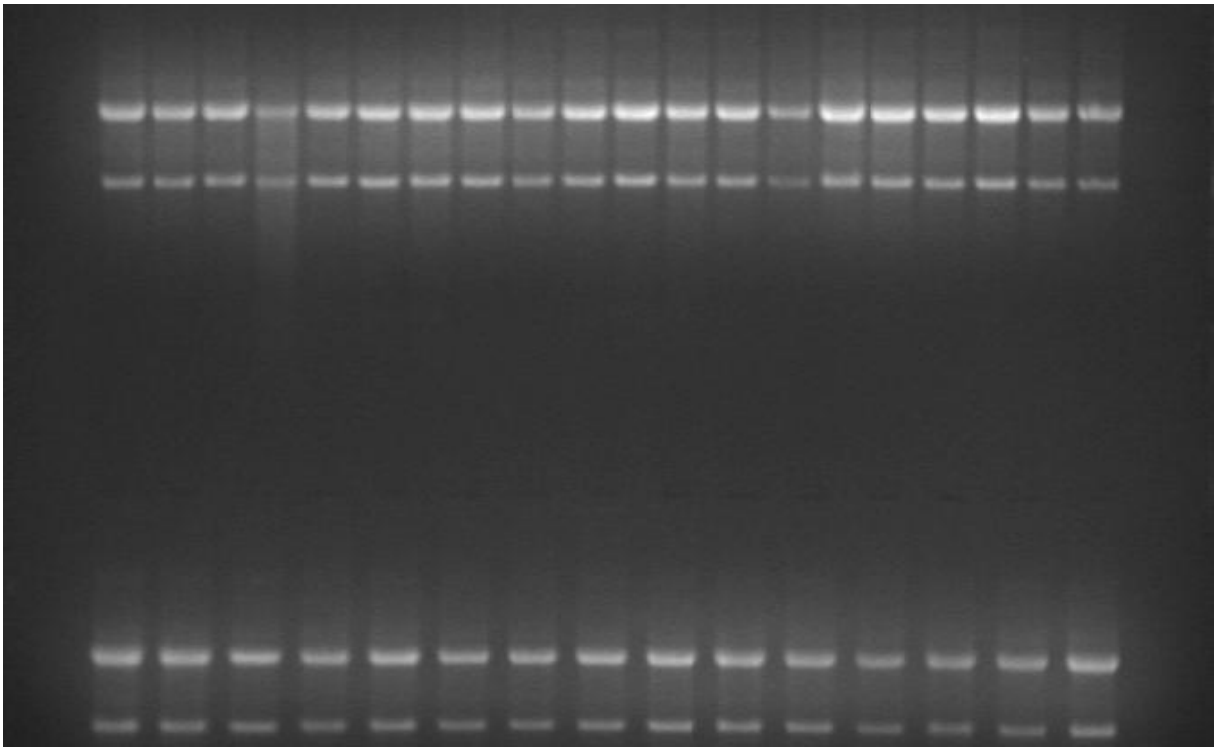

Figure 90: Photo of RNA agarose gel analysis

RNA (2 µg) from each condition was reverse-transcribed to cDNA (Superscript™ II Reverse Transcriptase, Invitrogen), and 20 ng of each cDNA template was used in PCR reactions with gene-specific primers. qPCR was performed in an MX3005p Multiplex Quantitative PCR system (Stratagene) using MESA Blue qPCR™ Mastermix Plus for SYBR® Assay Low ROX (Eurogentec SARL, Angers, France). We used one housekeeping/reference gene for normalization of the target genes, 18S Ribosomal 1 (S18). Corresponding *p* values were analyzed to evaluate the significance of each expression ratio after each PCR run using Student's t-test, for comparing the means of expression ratios between two conditions after three qPCR runs, which is equivalent to three biological replicates, for each tested target gene.

3. Western blot analysis

We prepared nuclear extracts. Briefly, thawed cells were washed twice in ice-cold PBS. The pellets were suspended in 1 mL of ice-cold buffer A (10 mM HEPES, pH 7.9,

1.5 mM $MgCl_2$, 10 mM KCl, 0.01% Triton X-100, 0.5 mM DTT, and 0.5 mM PMSF). After 20 minutes on ice, lysis was completed by vortexing the tube for 30 sec. Complete lysis was confirmed using trypan blue exclusion. Nuclei were recovered by centrifugation for 5 min at 5,000 rpm at 4°C and suspended in 25 µL of ice-cold buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 400 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, protease inhibitors (Complete-mini, Roche, Meylan, France) and 0.5 mM PMSF). Lysis of the nuclear membranes proceeded for 20 minutes on ice. Two cycles of freeze-thaw in liquid nitrogen for 30 seconds and incubation at 4°C for 5 minutes were performed. The extracts were centrifuged for 10 min at 13,000 rpm at 4°C, and the supernatant was recovered. Aliquots of 10 µL were stored frozen at -80°C.

For total extracts, cells were recovered by trypsinization and were lysed using CelLytic™ MT agent with a protease inhibitor cocktail, and then centrifuged at 1600 x g at 4°C for 10 min. The BCA kit (Interchim, Montluçon, France) was used to measure protein concentration, which was typically 1 mg/ml. For the analysis we used 20 µg of nuclear protein and 40 µg of total protein.

Protein samples, in SDS loading buffer, were electrophoretically separated by 10% SDS–PAGE and protein patterns were transferred, in Tris–glycine (25 mM Tris and 192 mM glycine) buffer containing 20% methanol, onto nitrocellulose membrane. Membrane was blocked, for 1 h at room temperature, with 5% fat-free milk powder in TBS (100 mM NaCl, 10 mM Tris–HCl (pH 7.8)) containing 0.1% Tween 20 and then incubated with either anti-GPx1 (1/1000), anti-TrxR1 (1/1000), anti-OGG1 (1/1000), anti-APE1 (1/3000), anti-TBP (1/5000) or anti-β-actin (1/10000) antibodies overnight at 4°C. The conjugated anti-IgG secondary antibody was then applied and band detection performed using the ECL kit. Each membrane was first used for detecting the target proteins (GPx1, TxrR1, OGG1, APE1,) and then deshybridized for monitoring the normalizing β-actin (for total extracts) or TBP (for nuclear extracts) protein on the same membrane. The intensity of each band was quantified with the

ImageJ software, and the ratio target protein/β-actin or /TBP calculated for each sample.

4. Determination of selenoenzyme activities

Cells were plated in culture flasks with or without SS or SM for 72 h. Cells were removed by trypsinization and were lysed using CelLytic™ MT (Sigma) agent with a protease inhibitor cocktail, and then centrifuged at 1600 x g at 4°C for 10 min. After a second centrifugation at 4800 x g at 4°C for 20 min, enzyme activities were measured in the supernatant. The protein content of the samples was determined by bicinchoninic acid assay (BCA) using a protein assay kit (Pierce) (Krieg et al., 2005). The results were expressed as mg/ml protein and used to normalize the value of the enzymatic activities to the amount of protein used in the assay.

Cytosolic GPx (GPx1) activity was measured in a reaction coupled with GR. The assay is based on the instant and continuous reduction of GSSG formed during GPx1 reaction by an excess of glutathione reductase (GR) activity providing for a constant level of GSH. As a substrate, t-butyl hydroperoxide was used and the concomitant oxidation of NADPH was monitored spectrophotometrically at 340 nm. One unit of enzyme was defined as the amount that converts 1 µmole of NADPH to NADP per min at 37 °C (Sigma).

Cytosolic TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit (Sigma). The method is based on the reduction of DTNB with NADPH into 5-thio-2-nitrobenzoic acid (TNB) the concentration of which is measured at 412 nm. One unit of TrxR activity was defined as the amount of enzyme that caused an increase in A_{412} of 1.0 per minute per mL at pH 7.0 at 25°C.

5. Cytotoxicity assay

LNCaP cells were plated in 35 mm plates or in 96-well microtitration plates (in 2 ml or 200 µl of cell culture media respectively) and were incubated at 37°C for 72h, in the presence of SS and SM.

Different agents were tested: UVA, UVB, UVC; γ- radiation; hydrogen peroxide (H_2O_2, added to the media for 30 min on ice); potassium bromated ($KBrO_3$, added to the media for 24 h at 37°C); methylmethane sulfonate (MMS, added to the media for 15 min at 37°C), Cis-Pt and bleomycin ($KBrO_3$, added to the media for 24 h at 37°C). The compounds were diluted in PBS (Ca^{2+} Mg^{2+}) whereas control cells were treated with PBS (Ca^{2+} Mg^{2+}) alone.

Immediately after exposure to the treatment, the culture media was changed and cells were incubated for 24 h hours. Cell viability was determined by the MTT assay; adding MTT in PBS (Ca^{2+} Mg^{2+}) solution (10% of the culture volume) to each well or plate. Cells were incubated with MTT for 2 h at 37°C, the media removed and DMSO (99.5% purity, equivalent to one of culture volume) was added to dissolve the formazan crystals. The optical density (OD) was measured at 565 nm using Multiscan RC microplate spectrophotometer (Labsystems, Helsinki, Finland). The OD of the blank (medium + MTT) was subtracted from all other OD values before calculation of cell viability.

6. Clonogenicity assay

Cell survival was assayed by the clonogenicity assay (or colony formation assay). LNCaP cells were plated in 35 mm plates and incubated at 37°C for 72 h with SS and SM. The cells were then stressed as described for the cell viability assay and immediately re-plated in a 60 mm plates at a density of 50 cell/mL. The culture medium was changed every 3 days. On the 12^{th} day medium was removed and crystal violet 0.5% in H_2O/MeOH (1:1) was added to each plate, left for 10 min under agitation and then carefully removed. The plates were rinsed with water, dried and the colonies were counted.

7. Alkaline single-cell gel electrophoresis (Comet Assay)

To evaluate the extent of DNA strand breaks by the Comet assay, LNCaP cells (50,000 cells/Petri dish 60 mm) were pre-treated with 30 nM SS or 10 μM SM for 72 h at 37°C and exposed to either 50 J/cm² UVA, 10 J/m²UVC, 200 μM H_2O_2 or 500 μM MMS. Control conditions were NT, SS and SM cells sham-treated with PBS. Immediately after treatment, cells were collected by trypsinization, washed once with PBS, counted and then suspended in freezing medium (20% FCS and 10% DMSO) at a concentration of $1x10^6$ cells/mL (aliquots of 500,000 cells), gently frozen to -80°C and stored in liquid nitrogen until use.

Frozen pellets of LNCaP (NT, SS, SM treated and the controls) were centrifuged at 300 x g for 5 min at 4°C. The pellets were suspended in PBS at a concentration of $2×10^6$ cell/mL. The cell suspension (50 μL) was added to 450 μl of agarose-low melting point at 37°C (final concentration 0.6% in PBS) and 100 μl of the mixture was placed on a microscope slide coated with one dried layer of 1% normal agarose in PBS. After gelling on ice**,** the slides were immersed overnight in cold lysis buffer (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris, 1% N-lauroylsarcosine sodium salt, 1% Triton X-100, 10% DMSO, pH 10;) at 4°C in the dark. Slides were washed 3×5 min with reaction buffer (40mM HEPES, 0.1M KCl, 0.5mM Na_2-EDTA, 0.2 mg/ml BSA). Digestion with lesion-specific glycoylases (100 μl per slide) was performed for 30 min at 37°C. Cells on control slides were incubated only with enzyme buffer. After incubation, slides were immersed in pre-cooled electrophoresis buffer (1 mM Na_2-EDTA and 300 mM NaOH) for 30 min in order to allow the unwinding of the DNA and then electrophoresed at 25 V/300 mA for 30 min. After electrophoresis, slides were rinsed three times for 5 min with neutralization buffer (0.4 M Tris-HCl, pH 7.4), stained with ethidium bromide (100 μl per slide, 2.5 μg/ml) and covered with a cover slip prior to analysis.

A fluorescence microscope (Carl Zeiss, Germany) connected to a charge-coupled device (CCD) camera and a computer-based analysis system (Comet assay IV software, Perceptive Instruments Ltd) was used to determine the extent of DNA damage after electrophoresis of DNA fragments in the agarose gel. For each

condition, 50 randomly selected cells were scored by slide, and triplicate slides were processed. The extent of damage was evaluated as the average of the triplicate values of the tail DNA intensity (% of DNA in the tail of the comet).

8. High pressure liquid chromatography-tandem mass spectrometry detection of DNA lesions

LNCaP cells (NT, SS and SM conditions) were irradiated in PBS in plastic petri dishes (100 mm diameter). DNA was extracted from cells using a sodium iodide (NaI) precipitation based chaotropic method. At first membrane cell was lysed with 750 µl of buffer A (320 nM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM deferoxamine, 1% triton X-100, pH7.5) and pellet was collected after centrifugation (1500 g for 5 min). Nuclear membrane was lysed with 300 µl of buffer B (5 mM EDTA-Na_2, 10 mM Tris-HCl, 0.15 mM deferoxamine, pH 8). After homogenisation, 15 µl of SDS 10% was added followed by 1.5 µl of RNAse A (100 mg/ml) and 3.5 µl of RNAse T1 (1 U/µl). the samples were incubated for 15 min at 50°C and the for 1h at 37°C after addition of 15 µl of protease (20 mg/ml). DNA was precipitated by addition of 600 µl of NaI solution (20 mM EDTA-Na_2, 7.6 M NaI, 40 mM Tris-HCl, 0.3 mM deferoxamine, pH 8) and 1 ml of isopropanol (100%). The samples were centrifuged for 5 min at 1500 g and the supernatant was removed and the pellets washed with 1 ml of isopropanol (40%). Another centrifugation was performed as previously after that 1 ml of ethanol was added. After a last centrifugation and complete elimination of ethanol, DNA was solubilised in 50 µL of deferoxamine. For DNA digestion, phosphodiesterase II (0.025 U), nuclease P1 (0.5 U) and DNAse II (2.5 U) were added to the DNA solution together with 2.5 µL of buffer (200 mM succinic acid, 100 mM $CaCl_2$, pH 6). The resulting solution was incubated for 2 h at 37°C,6 µL of buffer (500 mM Tris, 1 mM EDTA, pH 8) was added together with 0.003 units of phosphodiesterase I and 2 units of alkaline phosphatase, and incubated at 37°C for 2 h. In order to stop the reaction, 3.5 µL of HCl 0.1M was added to the mixture. The resulting solution was centrifuged (5000 x g) and the supernatant subsequently transferred into HPLC injection vials.

Samples were then analysed by high performance liquid chromatography associated to tandem mass spectrometry (HPLC-MS/MS) with electrospray ionization (Figure 91). The apparatus consisted in a SCIEX API 3000 electrospray triple quadrupolar spectrometer associated with a series 1100 Agilent chromatographic system. Quantification of DNA damage was performed in the multiple reactions monitoring (MRM) mode based on the isolation of a characteristic transition of the modified bases detected (Frelon et al., 2000).

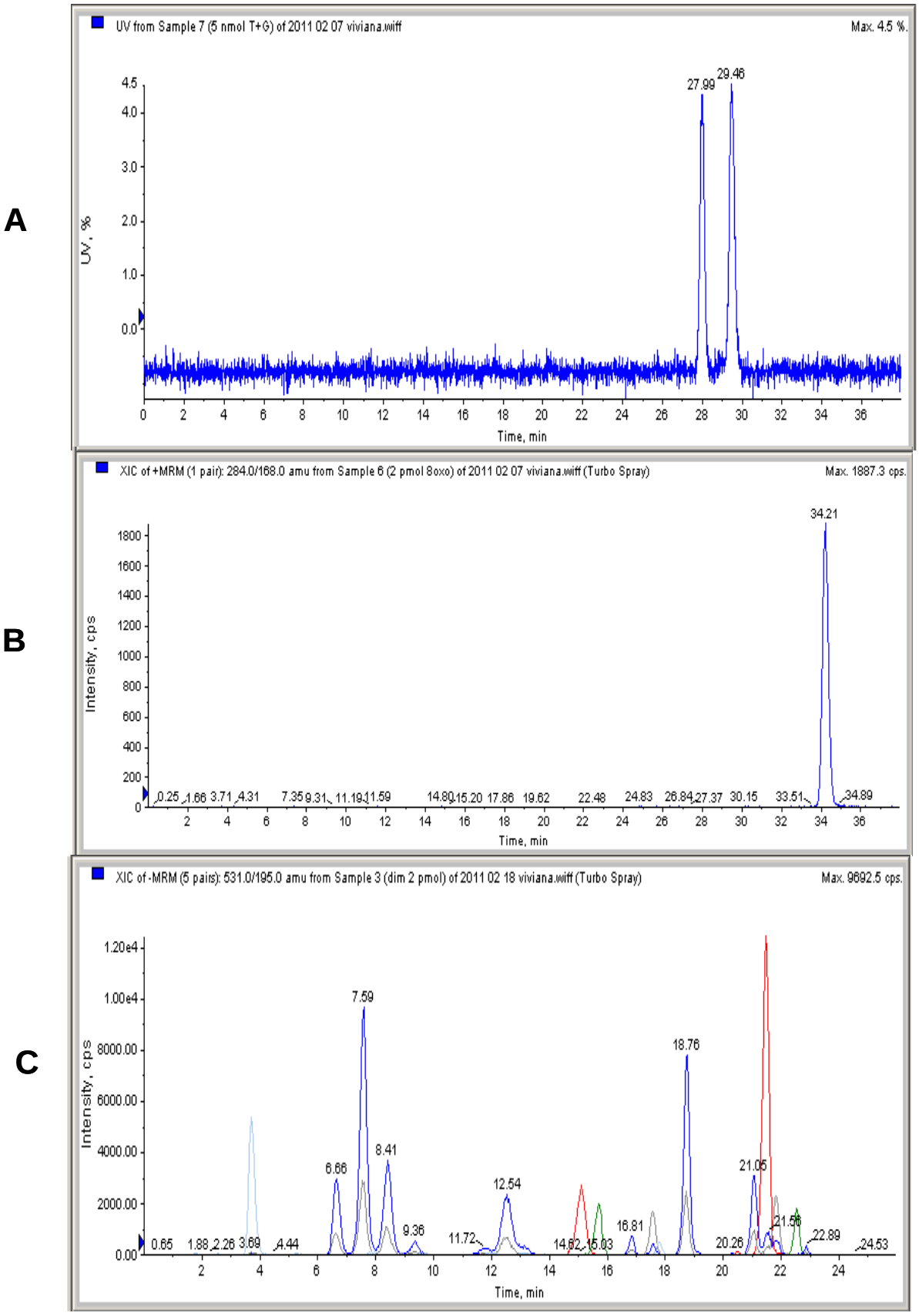

Figure 91: Example of LC-MS/MS analysis. (A) Detection of normal nucleosides (dG and Thd) by UV spectrometer; **(B)** MRM analysis of 8-oxoGua; **(C)** MRM analysis of different dimers.

9. Assessment of cellular DNA repair capacity in protein extracts

9.1 Substrate preparation

To evaluate DNA repair capacity by the Comet assay, the substrate cells were plated in 100 mm petri dishes and incubated at 37°C in 5% CO_2 for 24h h before use. The culture media was removed, and the cells were washed thoroughly with PBS.

Cells were exposed to an oxidative agent (Riboflavin 1µM + UVA 10 J/cm²) inducing 8oxoGua, an alkylating agent (MMS, 500µM) and a pyrimidine dimer-forming agent (UVC 10 J/m²), collected by trypsinization and suspended in freezing medium (RPMI-1640 with 20% FCS and 10% DMSO) at 5×10^5 cells/mL, slowly frozen to -80°C and stored in liquid nitrogen.

9.2 Preparation of whole cell extracts

Liquid nitrogen-stored frozen aliquots of LNCaP cells (NT, SS, SM conditions) were centrifuged at 300 X g, 4°C for 5 min, and the supernatant was removed, leaving a dry pellet that was subsequently frozen in liquid nitrogen and resuspended in 33 µL of extraction buffer (45mM HEPES, 0.4M KCl, 1mM EDTA, 10% Glycerol, 0.1 mM DTT, 0.25x Triton, pH 7.8). The mixture was then vortexed for 30 seconds, incubated for 5 min on ice and centrifuged at 14000 X g for 5 min at 4°C. The supernatant was then combined in a 1:5 ratio with reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/mL BSA, pH8). Extracts (50 µL) were placed on each slide covered with a cover glass and incubated for 30 min at 37°C. Samples digested with specific purified repair enzymes were used as positive controls while negative controls were incubated only with a 50 µL mix of extraction and reaction buffers. After digestion, the slides were transferred to an electrophoresis tank and processed using the Comet assay protocol described above (see section 7).

10. Oligonucleotide (ODN) biochip

10.1 Preparation of lesion ODN biochip

The multiplexed ODN array, which has been previously described (Sauvaigo et al., 2004), was used. Briefly, biotinylated support ODNs (at optimized concentrations: 1-

1.5 µM in PBS) were printed on streptavidin glass slides (Xantec bioanalytics GmbH, Germany) in duplicate in a 24-well format. The wells were subsequently individualized by setting the slides into ArrayIt® microplate hardware. Duplexes pre-formed through the specific hybridization of one Cy3-labeled lesion-bearing ODN and one long ODN were hybridized on support ODNs for 1 h at 37°C in a total volume of 80 µL. Each long ODN has a part complementary to a lesion ODN and a part complementary to a specific support ODN. This latter part directs the hybridization onto a specific location through the support ODN. Slides were then rinsed 3 times for 5 min with 80 µL of excision buffer (10 mM HEPES/KOH, pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM $ZnCl_2$, 1 mM DTT, and 0.5 mg/mL BSA).

Each well contained a control ODN and eight lesion-containing ODNs in duplicate: 8oxoG paired with cytosine (8oxoG-C), adenine paired with 8oxoG (A-8oxoG), thymine glycol paired with adenine (Tg-A), tetrahydrofuran as an AP site substrate equivalent paired with adenine (THF-A), hypoxanthine paired with thymine (Hx-T), dihydrothymidine paired with adenine (dHT-A), ethenoadenine paired with thimine (EthA-T) and uracil paired with adenine (U-A).

10.2 Excision reaction

On each 24-well slide we set 6 control wells containing excision buffer alone and 18 reaction wells for the excision reactions with the extracts. Nuclear extracts (20 µg/ml in 80 µl of excision buffer) were added to the wells in duplicate and incubated at 30°C for 30 minutes. The excision reaction was stopped by washing the slides 3 times for 5 min in PBS/0.2 M NaCl/0.1% Tween 20. The residual fluorescence of each spot was quantified at 532 nm using a Genepix 4200A scanner (Axon Instrument, Molecular Devices, Sunnyvale, CA, USA) and the Genepix Pro 5.1 software (Axon Instrument). Results were normalized as described previously (Millau et al., 2008). For each slide, we used the normalized fluorescence level of the control wells as a reference (repair buffer alone), and we set the fluorescence level of each lesion ODN of the control well to 100. The excision rate of each lesion was then calculated as a percentage of the fluorescence of the corresponding lesion ODN in the control wells. In addition, each well contained a control ODN (without any lesion) that was used to

assess the presence of any nonspecific degradation activity in the extracts. For the calculation of the final lesion ODN cleavage percentage, we then applied a correcting factor that took into account the possible control ODN degradation. Consequently, the final lesion ODN excision percentage was 100*(1-percentage of fluorescence of lesion ODN/percentage of fluorescence of control ODN).

The results are presented as the ratio of cleavage in treated vs. untreated cells for each lesion.

11. Competent bacterial cells

For competent cells, a fresh bacterial mini-culture was diluted 1/100 into 100 ml LB (without ampicilline antibiotic) and incubated at 37°C shaking incubator until OD 550 nm reached ~0.5. The culture was then chilled on ice (10 min), before being pelleted by centrifugation at ~3000 rpm for 5 min at 4°C. After removal of the supernatant, cells were resuspended in 10 ml filter sterilized TFB buffer (10mM KMES pH 6.2, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% (v/v) glycerol, 3mM $HACoCl_3$). After 10 min on ice, cells were centrifuged at ~3000 rpm for 5 min and resuspended in 2,4 ml filter sterilized cold TFB. Then, we added 84 µl of DMSO and incubated 25 min on ice. Aliquots (100 µl) were snap-frozen in liquid N_2 and stored at –80°C until required.

Ten µl of purified plasmid solution (maximum 10 ng) was added directly to 50 µl of thawed competent cell suspension. After incubation on ice for 30 min, cells were heat-shocked at 42°C water bath for 90 seconds before being immediately transferred back to ice for a further 5 min. The suspension was then plated out onto solid LB-agar plates supplemented with an appropriate antibiotic. Plates were inverted and incubated at 37°C overnight. Mini-cultures were prepared from selected colonies.

12. Plasmid Preparation

A single colony was picked from a freshly streaked selective plate and was inoculated in a starter culture of 2 ml LB medium containing the appropriate selective antibiotic. The incubation was carried out for ~8 h at 37°C with vigorous

shaking (~300 rpm). Then, we diluted the starter culture 1/500 into selective LB medium. For low-copy plasmids, we inoculated 500 ml medium, at 37°C for 12–16 h with vigorous shaking (~300 rpm). The plasmid purification was carried out by utilisation of MAXI-Prep Qiagen. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. 6000 x g. We removed all traces of supernatant and we resuspend the bacterial pellet in 10 ml Buffer P1, with RNase A addition. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

Subsequently, we added 10 ml of Buffer P2, mixing gently, and then we incubated at room temperature for 5 min. Then we added 10 ml of chilled Buffer P3, by inverting 4–6 times, and we incubated on ice for 15 min. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and SDS. To recover plasmid DNA, we centrifuged at 20,000 x g for 30 min at 4°C and we recovered the supernatant. We iterated the centrifugation step to ensure the recovery of all the supernatant containing plasmid DNA.

Then, we purified the plasmid DNA by a chromathographic separation using a QIAGEN-tip 500 column. We equilibrated the column by applying 10 ml of Buffer QBT, and we allowed the column to empty by gravity flow. The plasmid supernatant was added to the column and dropped off the resin column by gravity flow. The column was washed by applying 2 x 30 ml Buffer QC.

Finally, plasmid was eluted with 15 ml of Buffer QF. Then, plasmid was precipitated by adding 10.5 ml room-temperature isopropanol to the eluted DNA. The tube was centrifuged at 15,000 x g for 30 min at 4°C and we carefully decanted the supernatant. Plasmid pellet was washed with 5 ml of room-temperature 70% ethanol, and centrifuged at 15,000 x g for 10 min. The pellet was dried for 5–10 min, and then suspended in a suitable volume of water. To determine the yield, DNA concentration should be determined by nanodrop UV spectrophotometer and a quantitative analysis was carried out using an agarose gel analysis.

During this work we amplified four different plasmids: pGLuc, pEGFP, pGL4 and Renilla.

13. Host cell reactivation

LNCaP cells were plated and pre-treated with or without 30 nM SS or 10 µM SM for 72 h. Then, we collected and re-plated cells into 24-well plates. Previously, firefly luciferase reporter plasmid (pGLuc or pGL4) was damaged with different methods or not, such as by UVC radiation or Riboflavin coupled with UV visible light.

For the first stress, drops of plasmid solution (40 µl of a solution of 40 ng/µl) were deposited on a petri dish and frozen at -80°C. Once plasmids were frozen; we irradiated them with different doses of UVC. For the second stress, we prepared riboflavin solutions at different concentrations. For 100 µl of riboflavin solution 100 µl of plasmid (1 µg/µl) was added; then we irradiated with UV visible light for 2 min.

Undamaged Renilla luciferase (pRL-TK) reporter (Promega) was used as the internal control only when we used pGL4 plasmid. Plasmids were co-transfected into LNCaP cells with Lipofectamine LTX (Invitrogen). pGLUC plasmid were cotransfected with pEGFP and pGL4 with Renilla.

The luminescence was measured with Wallac a 1420 VICTOR2 multi-well spectrometer.

13.1 Lipofectamine optimization

In four microcentrifuge tubes labeled A–D, dilute DNA and medium without serum to obtain the final concentrations and volumes indicated below.

A) 398 µL of 5 ng/µL DNA and 2 µL Medium without serum
B) 396 µL of 10 ng/µL DNA and 4 µL Medium without serum
C) 394 µL of 15 ng/µL DNA and 6 µL Medium without serum
D) 392 µL of 20 ng/µL DNA and 8 µL Medium without serum

The mix solution was incubated for 10 min at room temperature.

Then, in a microcentrifuge tube labeled "LTX dilution," Lipofectamine™ LTX was diluted 1:10 in medium without serum (50 µL of Lipofectamine™ LTX to 450 µL of medium). From the lipofectamine diluted solution, we prepared different concentration of Lipofectamine with medium for a final volume of 50 µl. Table 7 below represents the different amount of lipofectamine for each wells.

	1	**2**	**3**	**4**	**5**	**6**
A	3.75 µL	5 µL	6.25 µL	7.5 µL	10 µL	12.5 µL
B	7.5 µL	10 µL	12.5 µL	15 µL	20 µL	25 µL
C	11.25 µL	15 µL	18.7 µL	22.5 µL	30 µL	37.5 µL
D	15 µL	20 µL	25 µL	30 µL	40 µL	50 µL

Table 7: volume of diluted Lipofectamine LTX

Then, we added for each 50 µl of the four plasmid solution prepared before into each well across the row corresponding to the label on the tube. We incubated the plate containing the plasmid-lipofectamine mixture for 30 minutes at room temperature. The entire 100 µL of this solution was transferred from each well to the appropriate well of the 24-well plate containing the cells in 500 µL of growth medium. We incubated cells for 24 h and then we measured the luminescence.

For all experiments we used 500 ng of plasmid and 2.5X ratio of lipofectamine to DNA, corresponding to the well B3.

13.2 BioLux® Gaussia Luciferase Assay Kit

At different post-transfection times (9, 24 and 48 h), we collected 40 µl of culture medium to measure luminescence. We prepared a 1X *Gaussia* Luciferase assay working solution by adding 50 µl of the GLuc Substrate to 5 ml of the GLuc Assay Buffer immediately before performing assay. The culture supernatant (20 µl) was placed into a sample tube and then 50 µl of the 1X GLuc assay working solution was added to the sample and promptly the luminescence was measured.

13.3 Dual-Luciferase® Reporter Assay

LNCaP cells pre-treated or no with selenocompounds (30 nM SS or 10 µM SM) were co-transfected with pGL4 and renilla plasmids into 24-well plate. We tested different ratio between pGL4 and Renilla 1:1, 5:1, 10:1 and 50:1. At different post-transfection times (9, 24 and 48 h), we removed growth media from cultured cells and rinsed then in 1X PBS. Then, we removed all rinse solution and 100 µl of 1X PLB (Passive Lysis Buffer) was dispensed into each well. 1X PLB (Passive Lysis Buffer). The culture vessel was gently shaken for 15 minutes at room temperature. Twenty µl of lysate was transferred to a 96-well black plate. Then we dispensed 100µl of LAR II (resuspend the lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II) and firefly luciferase activity was measured. After that we added 100µl of Stop & Glo® Reagent (add 50X Stop & Glo® Substrate to Stop & Glo® Buffer for a final 1X concentration) and Renilla luciferase activity was measured.

13.4 Dual-Glo® Luciferase Assay System

Alternatively to the Dual-Luciferase® Reporter Assay we also used Dual-Glo® Luciferase Assay System designed to be stable at room temperature for several hours.

We removed 24-well plates containing our different cells conditions from the incubator. To each plate well, we added a volume of Dual-Glo® Reagent equal to the volume of culture medium in the well and mix (75 µl of reagent for 75 µl of medium). The 24-well plate was gently shaken for at least 10 minutes to allow for cell lysis. We transferred 150 µl to each sample to 96- well black plate and then the firefly luminescence was measured. Then we added an equal volume of Dual-Glo® Stop & Glo® Reagent (dilute the Dual-Glo® Stop & Glo® Substrate 1:100 into Dual-Glo® Stop & Glo® Buffer) to the original culture medium volume to each well (75 µl). We waited at least 10 minutes and then measure Renilla luminescence was measured.

14. Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Statistical significances of differences among treatment groups were determined by Student's t-test. A p-value <0.05 was considered as statistically significant.

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ANNEXE 1

Résumé du travail de thèse

Le sélénium a été identifié par le physico-chimiste suédois Berzelius en 1817, au cours de recherches qu'il avait entrepris afin d'identifier l'agent responsable d'une maladie mystérieuse chez les ouvriers d'une usine pour la fabrication d'acide sulfurique. Cette découverte ayant eu lieu peu de temps après celle de l'élément tellurium, donnera le nom de la déesse grecque de la lune (sélène)à cet élément, sélénium.

Le sélénium (Se) a la particularité d'être à la fois un micronutriment essentiel mais également une substance toxique à fortes doses.

Ce n'est qu'à partir des années 50 que l'image du sélénium a évolué, pour arriver à son rôle essentiel qu'on lui connaît aujourd'hui. En 1954, il fut démontré que certaines bactéries se développaient plus rapidement dans un milieu enrichi en sélénium.

Toutefois, c'est à l'Allemand Klaus Schwarz que l'on doit, en 1957, la définition du sélénium comme un oligo-élément essentiel. Ses travaux ont démontré qu'une carence en sélénium pouvait entraîner une nécrose fatale du foie chez le rat. Dans les années 60, ce rôle essentiel fut confirmé chez plusieurs animaux d'élevage.

Au début des années 70, dans le cadre d'études chez l'homme, des scientifiques chinois ont suggéré que des carences en sélénium pouvaient être la source de deux pathologies graves, à savoir, les maladies de Keshan et de Kashin-Beck. Par la suite, il a été montré que des déficiences en sélénium étaient associées à des cas de retard mental, d'infertilité masculine, de déficience immunitaire, de maladies cardiovasculaires ainsi qu'à des myopathies et/ou cardiomyopathies. Ces mêmes carences sont également impliquées dans l'apparition de plusieurs types de cancers,

dans la recrudescence de certaines infections virales, ainsi que dans l'accélération des processus de vieillissement.

Les besoins journaliers en sélénium nécessaires à un homme adulte ont été estimés à 50-200 µg. Le seuil maximal d'apport journalier est évalué à 450 µg.

Les apports alimentaires en sélénium sont essentiellement fournis par les céréales, les féculents, les champignons, les viandes rouges et abats, le poisson et les œufs, dont les teneurs en sélénium varient en fonction de la disponibilité et de l'abondance de l'oligo-élément dans l'environnement d'origine. Toutefois, cette dose minimale et surtout les formes sous lesquelles elles sont apportées sont l'objet de nombreux débats, notamment en ce qui concerne les supplémentations alimentaires.

L'efficacité d'absorption du sélénium est élevée (50 à 95 %). Elle dépend de la forme d'apport du sélénium, du statut adéquat ou non en sélénium et de la présence ou non d'autres aliments. La sélénométhionine, mieux absorbée que le sélénite, l'est par un mécanisme actif analogue à celui de la méthionine. Le sélénite est absorbé par simple diffusion mais son absorption est stimulée par la présence de nutriments à groupements thiols comme la cystéine ou le glutathion. L'ensemble des formes organiques et inorganiques du sélénium peuvent être utilisées par l'organisme mais leur métabolisme est différent.

Le sélénium absorbé est réduit à l'état de séléniure puis incorporé dans les protéines sous forme de sélénocystéine (Sec) par l'intermédiaire d'un ARNt spécifique. Cet acide aminé, considéré comme le 21ème acide aminé, a la particularité d'être codé par un codon UGA, normalement considéré comme un codon stop. Son incorporation dans les protéines a lieu par un mécanisme complexe mais original qui inclut cet acide aminé de manière co-traductionnelle, en utilisant un codon stop dont le sens est changé par une structure particulière en tige-boucle au niveau de l'ARNm qui permet que le codon UGA soit traduit comme Sec. Chez les eucaryotes, la structure tige-boucle se trouve dans une région 3' non traduite de l'ARN messager à une certaine distance du codon UGA. La structure en tige-boucle (SECIS) va être au centre d'un complexe étroit formé d'une protéine de liaison (SBP2), un facteur d'élongation spécifique (EFsec) qui va permettre de présenter au site ribosomal

l'ARNt^{Sec}, transformant le codon stop, présent à ce moment dans le ribosome, en codon Sec. Cet ARNt a, lui aussi, été chargé d'une sélénocystéine de manière très particulière, puisque des enzymes vont transformer un ARN de transfert ARNt^{Sec}, qui possède des caractéristiques de structures secondaires et tertiaires distinctes des ARNt habituels et qui est chargé d'une sérine, en ARNt chargé d'une Sec.

L'expression des sélénoprotéines est dépendante de certains organes ou tissus, fonction du statut en sélénium et de la forme d'apport en sélénium. En cas de restriction en sélénium, les concentrations des sélénoprotéines sont diminuées avec cependant un maintien de certaines sélénoprotéines dans certains tissus bien spécifiques (cerveau, organes de la reproduction, glandes endocrines). A aujourd'hui environ 25 sélénoprotéines ont été identifiées, certaines desquelles ont un rôle biologique encore inconnu.

Le sélénium joue un rôle de cofacteur biologique de certains enzymes antioxydants, notamment la glutathion peroxydase et la thioredoxine réductase, deux enzymes précieuses pour nous protéger contre l'oxydation par les radicaux libres. Plusieurs études montrent qu'une augmentation du sélénium dans le régime alimentaire pouvait être bénéfique contre le cancer du foie, du colon, du pancréas et surtout de la prostate. Une des premiers études épidémiologiques sur le sélénium était le Nutritional Prevention of Cancer trial, commencé en 1996 par Clark et al.. Elle a montré que la supplémentation alimentaire avec 200 µg/jour de sélénium conduit à une réduction de 63% du risque de cancer de la prostate(Duffield-Lillico et al., 2003). Par ailleurs, les chiens et les humains sont les deux seules espèces pour lesquelles le cancer de la prostate survient spontanément avec une certaine fréquence.

La plupart des auteurs explique cet effet par son rôle antioxydant et immunostimulant mais ces mécanismes sont loin d'expliquer tous les effets préventifs du sélénium sur le cancer. Une hypothèse serait que les effets du sélénium aient lieu en amont de la tumorogenèse, dans la prévention de l'apparition de mutations délétères, grâce aux mécanismes de protection contre les agressions oxydantes mutagènes de l'ADN.

Cependant, le mécanisme moléculaire d'action du sélénium reste largement inconnu. L'objectif de cette étude était d'étudier l'effet des faibles concentrations de deux composés du sélénium sur la capacité de réparation de l'ADN dans les cellules du cancer de la prostate LNCaP. Le sélénium pourrait diminuer l'incidence des mutations par augmentation de la réparation et/ou diminution de la formation des dommages de l'ADN.

Pendant ce travail de thèse, les cellules humaines du cancer de la prostate (LNCaP) ont été utilisées comme un outil *in vitro* pour évaluer le potentiel du stress oxydant et la génotoxicité de plusieurs agents de stress. D'autre part, la protection de la prostate contre le cancer par le sélénium a été bien documentée et, par conséquent les cellules LNCaP sont un bon modèle *in vitro* pour évaluer son rôle protecteur.

Nous avons utilisé deux différents composés du sélénium, le sélénite de sodium (SS) d'origine inorganique et la sélénométhionine (SM) d'origine organique; ces deux composés ont un métabolisme cellulaire différent.

Une supplémentation en sélénium peut augmenter le taux de certaines sélénoprotéines qui jouent en rôle antioxydant. Donc, nous avons analysé l'expression et l'activité de deux selenoenzymes, la glutathion peroxydase (GPx1) et la thiorédoxine réductase (TrxR).

Avant d'évaluer les dommages à l'ADN causés par l'exposition à plusieurs stress, il était nécessaire de déterminer la concentration optimale de SS et SM pour le traitement des cellules et l'étude de la cytotoxicité de différents agents de stress. Ces derniers on été choisis afin de tester une grande variété d'agents qui induisent différentes classes de dommages.

Les concentrations optimales de SS et SM utilisées sont très faibles et très différentes entre les deux composés; la concentration de SM (10 µM) est 300 fois plus concentrée que SS (30 nM).

Ensuite, nous avons évalué la protection du traitement avec le sélénium contre l'endommagement de l'ADN par différents agents. Ces résultats ont mis en évidence que le traitement avec les deux composés du sélénium permettait une meilleure

survie cellulaire après un stress de type oxydatif. Par ailleurs, le traitement avec SS ou SM réduit la formation de lésions oxydées à l'ADN par rapport aux cellules sans sélénium.

Ensuite, nous nous sommes concentrés sur l'étude des capacités de réparation de l'ADN dans les cellules LNCaP prétraitées avec les deux composés du sélénium.

Dans un premier temps, nous avons analysé le taux de réparation de l'ADN par une étude cinétique. Nous avons utilisé le test des comètes afin de déterminer le taux de réparation des différents dommages induits. Les cellules prétraitées avec SS et SM ont montré une plus grande capacité de réparation des dommages oxydatifs, en particulier de la 8-oxoG.

La différente vitesse de réparation entre les cellules avec ou sans sélénium nous a amené l'idée de déterminer le niveau d'expression de certaines enzymes de réparation impliqués dans le système de réparation BER. A l'état basal les cellules prétraitées avec SS sur-exprimait l'enzyme impliquée dans la réparation de la 8-oxoGua, hOGG1. Les intéressants résultats obtenus nous ont conduits à évaluer également l'expression au niveau des protéines de ces enzymes.

Enfin, nous avons utilisé deux tests complémentaires afin d'évaluer la capacité d'excision des extraits protéiques, prétraités ou pas avec le sélénium. Le premier se base sur le test des comètes modifiés qui permet la mesure de l'activité d'excision des extraits protéiques sur l'ADN génomique; le deuxième en utilisant une biopuce oligonucleotidique pour quantifier simultanément l'activité de plusieurs glycosylases présentes dans les extraits nucléaires.

La dernière partie de cette étude a été dédiée à l'optimisation du test HCR (Host Cell Reactivation), un outil de biologie moléculaire qui permette de mesurer les activités de réparation de l'ADN *in cellulo.* Le principe est basé sur la transfection des cellules, dites hôtes, avec un plasmide portant le gène rapporteur d'une protéine luminescente. Le plasmide a été préalablement inactivé à cause de la présence de dommages induits par un traitement chimique. La capacité de la cellule de réparer les dommages présents sur le plasmide permet la réactivation du gène rapporteur qui peut être évalué par luminescence.

Différentes voies de réparation de l'ADN peuvent être analysés par cette technique en endommageant le plasmide rapporteur de différentes manières. Le plasmide luciférase a été endommagé par un traitement Riboflavine + lumière visible afin d'induire une formation sélective de la 8-oxoGuanine. Cette lésion est prise en charge par la voie de réparation BER. Les résultats préliminaires montrent que les cellules prétraitées avec le sélénium réparent plus rapidement le plasmide. Jusqu'à présent, il n'y a aucun travail avec le technique de l'HCR qui utilise un plasmide enrichi en 8-oxoGua. Par ailleurs, nous allons effectuer plusieurs analyses cinétiques d'évaluer l'évolution de la réparation du plasmide.

Cette étude nous a donné une vision plus mécanistique de l'influence du prétraitement avec le sélénium sur les dommages et la réparation de l'ADN. Par ailleurs, nous comprenons mieux quel système de réparation de l'ADN est affecté par le sélénium dans le modèle cellulaire adopté. En plus, les cellules prétraitées avec le sélénium présentaient une activité de réparation accrue envers les dommages oxydatifs, indiquant un nouveau mécanisme d'action du sélénium. Notre travail fournit une vue étendue de la protection conférée par le sélénium contre le cancer: le sélénium, non seulement se comporte comme un antioxydant, mais aussi améliore la capacité de réparation de l'ADN.

En conclusion, nous pouvons imaginer que le mécanisme d'action protecteur du sélénium est représenté par un délicat équilibre entre l'activation et la répression de l'activité de certaines protéines plus ou moins directement impliquées dans la réparation de l'ADN et la progression de la croissance cellulaire.

ANNEXE 2

Conferences participation

- **Oxidative DNA damage: from chemical aspects to biological consequences:** 8th Winter Research Conferences at the Centre de l'Ecole de Physique of Les Houches, France (17-23 January 2009): **oral presentation**

- **4èmes Journées Scientifique du CLARA** (24-25 march 2009, Archamps France) : poster presentation and **winner of the Poster Prix**

- **Gordon Research Conference on Genetic Toxicology,** New Hampshire USA (9-14/08/2009, New London): **poster presentation**

- **5èmes Journées Scientifiques du CLARA,** Lyon France (30-31 march 2010) **: poster presentation**

- **Photobiology 3rd joint meeting of the French and the Italian Societies for Photobiology** (25-26 october 2010) in Paris France: **poster presentation**

- **4ème Symposium International Nutriotion, Biologie de l'Oxygène et Médicine** (15-17 june 2011) in Paris France: **poster presentation**

Scientific productions

- **Inverse relationship between serum bilirubin and oxysterols, sensitive markers of oxidative stress**

  Libor Vitek[1], Ladislav Novotny[1], Ales Zak[1], Tomas Zima[1], Roberto Monticolo[2], **Viviana De Rosa**[2], Luigi Iuliano[2]

  Hepatology International (2008) Volume 2, Supplement 2, 37-211, DOI: 10.1007/s12072-008-9079-9

  [1] *Charles University in Prague, Czech Republic,* [2] *Sapienza University, Italy*

- **Effect of selenium on lethal and genotoxic properties of oxidative stress**

  Viviana De Rosa, Diamond Alan, Favier Alain, Douki Thierry, Rachidi Walid

  Bulletin du cancer, Volume 97 numéro spécial mars 2010 Document Type: Meeting Abstract

- **Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells.**

  Erkekoğlu P, Rachidi W**, De Rosa V**, Giray B, Favier A, Hincal F.

  Free Radic Biol Med. 2010 Aug 15;49(4):559-66. Epub 2010 May 11.

  INAC/SCIB/LAN, CEA de Grenoble, 17 rue des Martyrs, 38054 Grenoble, France

- **Selenium protects against the cytotoxic and genotoxic effects of oxidative stress both as an anti-oxidant and by stimulating DNA damage repair**

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  Bulletin du cancer Volume: 98 (S92-S92) Suppl.1 mars 2011, Document Type: Meeting Abstract

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Pre-accepted in the journal Free Radical Research:

- **Selenium supplementation specifically stimulates the repair of oxidative DNA damage**

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Submitted:

- **Alzheimer's disease-associated neurotoxic peptide amyloid-β impairs base excision repair in human neuroblastoma cells**

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ANNEXE 3

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Original Contribution

Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells

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ABSTRACT

Selenium is an essential cofactor in the key enzymes involved in cellular antioxidant defense. It plays a critical role in testis and reproduction and regulates DNA damage within the prostate. Phthalates are ubiquitous environmental contaminants that cause alterations in endocrine and spermatogenic functions in animals. The objective of this study was to investigate the cytotoxicity and genotoxicity potentials of di(2-ethylhexyl)phthalate (DEHP), the most widely used phthalate and its primary toxic metabolite mono(2-ethylhexyl)phthalate (MEHP), and their effects on the antioxidant balance in the LNCaP human prostate adenocarcinoma cell line. Protection by selenium supplementation with either sodium selenite (SS, 30 nM) or selenomethionine (SM, 10 μM) was also investigated. Both DEHP (3 mM) and MEHP (3 μM) caused significant decreases in cell viability; altered antioxidant status, particularly decreasing the GPx1 activity; and induced DNA damage as measured by the alkaline comet assay. Selenium supplementation was highly protective against cytotoxicity, partially prevented genotoxicity, and restored the antioxidant status. The results of this study suggested that the underlying mechanism of cytotoxicity and resulting disturbances produced by DEHP or MEHP was an an oxidative stress process and/or an effect on the expression of antioxidant enzymes, and accentuated the importance of selenium status, particularly with respect to the high probability of phthalate exposures and their adverse effects.

Introduction

Oxidative stress plays an important role in the modulation of several important physiological functions, but also accounts for changes that can be detrimental to the cells [1]. Currently, there is a growing interest in environmental chemicals that can cause oxidative stress in the reproductive system resulting in disturbances of spermatogenesis [2], besides the association of oxidative stress with degenerative diseases including cancer [3].

The essential trace element selenium (Se) is involved in fundamental biological processes ranging from cellular antioxidant defense to the protection and repair of DNA, and apoptosis [4]. Low dietary Se intake makes the organism prone to oxidative stress-related conditions, reduced fertility and immune functions, and increased risk of cancers [5]. Human studies have indicated that Se supplementation in a population with low basal blood Se levels decreases the incidence of several types of cancers, particularly of prostate [6]. Se is therefore considered as a promising chemoprotective agent for prevention of prostate cancer which is the most common malignancy and one of the leading causes of cancer-related death in men [6]. Se, with its several forms of cellular selenoproteins, is involved in the modulation of intracellular redox equilibrium [7]. High concentrations of Se induce apoptosis in LNCaP human prostate cancer cells in association with production of reactive oxygen species (ROS), alteration of cell redox state, and mitochondrial damage [8]. LNCaP cells express prostate-specific antigen (PSA), p53, peroxisome proliferator-activated receptor α (PPARα), and peroxisome proliferator-activated receptor γ (PPARγ) [9]. Therefore, the LNCaP cell line is a good *in vitro* model for assessing

Abbreviations: BCA, bicinchorinic acid assay; CDNB, 1-chloro-2,4-dinitrobenzene; DEHP, di(2-ethylhexyl)phthalate; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; FCS, fetal calf serum; GPx1, glutathione peroxidase 1; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; H_2O_2, hydrogen peroxide; H_2Se, hydrogen selenide; LNCaP, lymph-node-derived androgen-sensitive cell line; MEHP, mono(2-ethylhexyl)phthalate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Na_2-EDTA, disodium ethylenediaminetetraacetic acid; NaCl, sodium chloride; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NaOH, sodium hydroxide; p53, protein 53; PBLs, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PP, peroxisome proliferating; PPARγ, peroxisome proliferator-activated receptor γ; PPARα, peroxisome proliferator-activated receptor α; PSA, prostate-specific antigen; Rb, retinoblastoma; ROS, reactive oxygen species; Se, selenium; SEM, standard error of mean; SM, selenomethionine; SS, sodium selenite; TNB, 5-thio-2-nitrobenzoic acid; TrxR, thioredoxin reductase.

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the oxidative stress potential and genotoxicity of peroxisome-proliferating (PP) environmental chemicals such as phthalates, besides its responsiveness to Se.

As the most abundantly used additives in plastics, phthalates are ubiquitous environmental chemicals, act as hepatocarcinogens in rodents [10], and are known as endocrine disruptors that target the fetal and pubertal testis and lead to alterations in endocrine and spermatogenic functions [11]. Di(2-ethylhexyl)phthalate (DEHP) is the most important phthalate with respect to its production, use, and occurrence in the environment. It is mainly used in polyvinyl chloride plastics in the form of numerous consumer and personal care products and medical devices. DEHP induces testicular damage in both developing and adult animals [11,12], and its main metabolite, mono (2-ethylhexyl)phthalate (MEHP), is also involved in its toxic effects, resulting in testicular damage and decreased sperm motility [13]. The mechanisms by which phthalates and specifically DEHP exert toxic effects in the reproductive system are not yet fully elucidated. Some of the effects are related to their antiandrogenic potential [12,14]. A PPARα-mediated pathway based on their PP activity [15] and activation of metabolizing enzymes leading to free radical production and oxidative stress have also been suggested [16]. On the other hand, the genotoxic potential of DEHP and MEHP has been demonstrated previously in different tissues and with various genotoxicity assays [17,18].

Taking together the essentiality of Se for cellular antioxidant defense, frequency of inadequate Se intakes, and high probability of DEHP exposure in humans, this study was designed to investigate the potential of DEHP and MEHP to cause intracellular antioxidant imbalance, cytotoxicity, and genotoxicity in human prostate cells, using the LNCaP human prostate adenocarcinoma cell line as a model. The ultimate objective of this study was to investigate whether Se supplementation in the form of sodium selenite (SS) or selenomethionine (SM) would be protective.

Materials and methods

Chemicals and reagents

MEHP was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The protein assay kit was from Uptima Interchim (Montluçon, France). NaOH was purchased from Carlo Erba (Rodano, Italy). RPMI 1640 medium and fetal calf serum (FCS) were from GIBCO (Courbevoie, France). All other chemicals including DEHP, SS, SM, dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-chloro-2,4 dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB); colorimetric assay kits for thioredoxin reductase (TrxR), glutathione reductase (GR), and glutathione (GSH) measurements; Cell Lytic M cell lysis reagent and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and treatment

The LNCaP human prostatic cancer cell line (lymph-node-derived androgen-sensitive cell line, normal for cell-cycle-related tumor suppressor genes p53 and retinoblastoma (Rb), [Wild type]) was a gift from Prof. Alan Diamond, University of Illinois (IL,USA). Cells were maintained in RPMI 1640 medium containing 5% FCS, at 37 °C in a humidified incubator under 5% CO_2, and cultured by plating onto 96-well microtiter plates using RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin at 37 °C.

SS and SM stock solutions were prepared in sterile, deionized water. DEHP (50 mM) and MEHP (100 μM) stock solutions were prepared in 0.1% DMSO, and fresh dilutions were made using culture medium to achieve final concentrations ranging from 1 μM to 10 mM for DEHP, and 1 to 30 μM for MEHP. Cell viability measurements were performed in LNCaP cells incubated with various concentrations of DEHP or MEHP for 24 h. For the assessment of the protective effects of Se, LNCaP cells supplemented with 30 nM SS or 10 μM SM were cultured for 72 h, and then exposed to various concentrations of DEHP or MEHP for 24 h while continuing the Se supplementation. The doses of Se in the form of SS and SM used in this study were chosen from preliminary experiments (not shown) as concentrations do not inhibit cell growth and do not cause cytotoxicity, but result in maximal GPx1 induction after 72 h of incubation.

For the measurement of antioxidant enzyme activities and glutathione levels, and for comet assay the following treatment groups of LNCaP cells were prepared: Nontreated cells (NT-C), LNCaP cells were cultured without any treatment for 24 h; SS-supplemented cells (SS-S), LNCaP cells were cultured with 30 nM SS for 72 h; SM-supplemented cells (SM-S), LNCaP cells were cultured with 10 μM SM for 72 h; DEHP-treated cells (DEHP-T), LNCaP cells were cultured with 3 mM DEHP for 24 h; DEHP-treated SS-S cells (SS/DEHP-T), SS-S cells were cultured with 3 mM DEHP for 24 h; DEHP-treated SM-S cells (SM/DEHP-T), SM-S cells were cultured with 3 mM DEHP for 24 h; MEHP-treated cells (MEHP-T), LNCaP cells were cultured with 3 μM MEHP for 24 h; MEHP-treated SS-S cells (SS/MEHP-T), SS-S cells were cultured with 3 μM MEHP for 24 h; MEHP-treated SM-S cells (SM/MEHP-T), SM-S cells were cultured with 3 μM MEHP for 24 h.

Determination of cell viability

Cell viability was measured by a modified MTT assay [19]. Three thousand cells per well were plated onto 96-well microtiter plates in 200 μl medium with or without DEHP, MEHP, SS, or SM. After incubation for specified times at 37 °C in a humidified incubator, 20 μl of MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well. Medium was removed 2 h later, 100 μl of DMSO was added to dissolve the formazan product, and the absorbance was read at 570 nm using a Multiscan Ascent microtiter plate reader (Labsystems, France). The absorbance was proportional to viable cell number, and survival was calculated as the percentage of the staining values of untreated cultures. The percentage viability was calculated as "% specific viability = $[(A–B)/(C–B)]/100$", where A = absorbance of the treated cells at 570 nm, B = absorbance of the medium at 570 nm, and C = absorbance of the control cells at 570 nm.

Antioxidant enzyme activities and glutathione levels

After specified incubation periods and trypsinization, cells were lysed using Cell Lytic M Cell Lysis agent with a protease inhibitor cocktail, and then centrifuged at 4000 rpm, 4 °C, for 10 min. After further centrifugation at 13,000 rpm, 4 °C, for 20 min, antioxidant enzyme activities and GSH levels were measured in the supernatant.

The activity of cytosolic GPx (GPx1) was measured in a coupled reaction with GR as described by Flohé et al. [20]. The assay is based on the instant and continuous reduction of GSSG formed during GPx1 reaction by an excess of GR activity providing for a constant level of GSH. As a substrate, *t*-butyl hydroperoxide was used and concomitant oxidation of NADPH was monitored spectrophotometrically at 340 nm. One unit of enzyme was defined as the amount of GPx1 that transforms 1 μmol of NADPH to NADP per minute at 37 °C.

Cytosolic TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit as described previously [21]. The method is based on the reduction of DTNB with NADPH to 5-thio-2-nitrobenzoic acid (TNB) that is measured at 412 nm. One unit of TrxR activity was defined as the enzyme that caused an increase in A_{412} of 1.0 per minute per milliliter at pH 7.0 at 25 °C.

The activity of GR was measured by a Glutathione Reductase Assay kit, based on the reduction of oxidized glutathione (GSSG) by NADPH

in the presence of GR. One unit of enzyme was defined as the enzyme activity that caused the reduction of 1 μmol of DTNB at 25 °C at pH 7.5 [22].

Cytosolic glutathione-*S*-transferase (GST) activity was determined according to the method of Habig et al. [23] using CDNB as a substrate and measuring the change in absorbance at 340 nm. The results were given as nanomoles per minute per milligram protein.

For the measurement of the total GSH levels, cells were diluted with 5-sulphosalicylic acid for protein precipitation, and centrifuged at 4000 rpm, 4 °C, for 10 min. Supernatants were used for total GSH determinations by using the Glutathione Assay kit. The assay was based on the reduction of DTNB by NADPH by a reaction catalyzed by GR using GSH at 412 nm [24]. The results were given as picomoles GSH per milligram protein.

Protein content of the samples was determined by bicinchorinic acid assay (BCA) using a protein assay kit [25]. The results were given as milligrams per milliliter protein.

Alkaline single-cell gel electrophoresis (comet assay)

DNA damage was evaluated using the alkaline single-cell gel electrophoresis technique (the comet assay) that allows the measurement of single- and double-strand breaks together with alkali-labile sites. The assay was performed as described earlier [26,27] and measurements were made in two consecutive days on triplicate slides and the results were given as the mean value of 2 days. Immediately after the treatments, the cells were isolated, washed, and resuspended in PBS at a density of $\sim 2.5 \times 10^6$ cells/ml. Fifty microliters of this suspension was mixed with 450 μl solution of (0.6% in PBS) low melting point agarose, and 100 μl of the solution was spread on microscope slides covered with 1% agarose. Cells were lysed (2.5 M NaCl, 0.5 M Na_2-EDTA, 10 mM Tris, 1% sodium lauryl sulfate, 1% Triton X-100, 10% DMSO, pH 10) at 4 °C in the dark for 1 h. After lysis, cells were immersed in freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA, pH 13) for 30 min to allow DNA unwinding. Electrophoresis was then performed at 25 V/300 mA for 30 min. Slides were rinsed three times for 5 min with neutralization buffer (0.4 M Tris-HCl, pH 7.4), and stained with ethidium bromide (20 μg/ml) in PBS. For quantification, a fluorescence microscope (Carl Zeiss, Germany) was used which was connected to a charge-coupled device (CDC) and a computer-based analysis system (Comet Assay IV software, Perceptive Instruments Ltd), and the extent of DNA damage was determined after electrophoretic migration of DNA fragments in the agarose gel. For each condition 50 randomly selected comets on each slide were scored, and tail % intensity (percentage of DNA in the tail) and tail moment (product of comet length and tail intensity) were determined as an average of triplicate slides for each condition.

Statistical analysis

The data were expressed as mean ± standard error (SEM). Statistical significances of differences among treatment groups were determined by use of one-way analysis of variance and covariance (ANOVA), followed by Student's *t* test using a Statistical Package for Social Sciences Program (SPSS). A P value <0.05 was considered as statistically significant.

Results

Selenium supplementation increases resistance to DEHP and MEHP cytotoxicity

Cell viability was measured by the MTT assay in LNCaP cells exposed to various concentrations of DEHP or MEHP, and results are illustrated in Fig. 1 as relative to zero dose of DEHP or MEHP. As shown in Fig. 1A, DEHP had a flat dose–cell viability response curve. That is, at 1 μM concentration of DEHP, LNCaP cells survived 100%, and continued to show ~60 to 40% survival at a wide concentration range of 5 μM to 3 mM DEHP, whereas MEHP was toxic at the micromolar dose range showing a very steep dose–response curve (Fig. 1B). So that, at 5 μM MEHP concentrations, cell survival was ~20%, and there was no survival at concentrations ≥10 μM (Fig. 1B). Thus, the cytotoxicity of the MEHP, the metabolite of DEHP, was much higher than that of the parent compound.

When Se-supplemented LNCaP cells were exposed to DEHP, almost complete survival was observed for concentrations up to 50 μM (Fig. 1A). The protection by either SS (30 nM) or SM (10 μM) supplementation continued up to 3 mM DEHP concentration providing a level of ~80% survival. Whereas in 3 μM MEHP-exposed cells ~60% survival was observed, and Se supplementation provided complete survival (Fig. 1B). Protection by Se against cytotoxicity of MEHP decreased, however, with higher doses.

From these data, the doses of DEHP and MEHP to be used for the antioxidant status measurements and comet assay were chosen as 3 mM for DEHP (~40% survival dose) and 3 μM (~60% survival dose) for MEHP.

DEHP and MEHP impair antioxidant system and selenium supplementation provides significant restoration

The results of the enzymatic and nonenzymatic antioxidant measurements are summarized in Table 1. Se supplementation of LNCaP cells with either SS or SM (SS-S and SM-S groups) significantly increased the activities of TrxR (~3-fold), GPx1 (≥2-fold), and GR (~2-fold) compared to nontreated cells (NT-C), but did not cause any change in the total GSH level and GST activity.

TrxR activity did not change in LNCaP cells when exposed to 3 mM DEHP. Similarly, TrxR activity was not significantly different in SS/DEHP-T cells compared to SS-S cells. However, the activity observed in SM/DEHP-T cells was 55% less than that of SM-S cells. These results, thus, showed that Se either in SS or SM form induced the same level of upregulation in TrxR activity, but SM was not as effective as SS in maintaining the activity of TrxR in the presence of DEHP. Whereas exposure of LNCaP cells to 3 μM MEHP caused a significant increase (~40%) in the activity of TrxR compared to that of NT-C, exposure to MEHP in Se-supplemented cells (SS/MEHP-T and SM/MEHP-T cells) was not found to change the activity of TrxR significantly compared to SS-S or SM-S.

GPx1 activity significantly decreased in both DEHP-T and MEHP-T cells (>3-fold, and >4-fold, respectively) compared to NT-C; however, there was no significant difference between the effects of the two phthalate derivatives. Se supplementation with either SS or SM effectively countered the effect of DEHP by completely restoring the activity up to the control level (NT-C) or even higher. However, GPx1 activity remained lower than those of SS-S and SM-S cells in both SS/DEHP-T and SM/DEHP-T groups, indicating that DEHP interferes with the upregulation of GPx1 by Se in LNCaP cells. In the case of MEHP treatments, both SS and SM supplementations significantly restored the effect of 3 μM MEHP on GPx1 activity, providing ~2-fold increase. But, as was with DEHP exposure, the activity of GPx1 remained much lower (~2- to 2.5-fold) than that of NT-C and much more lower than those of SS-S and SM-S cells.

None of the treatments caused a change in GST activity. Similarly, total GSH levels did not change with either DEHP or MEHP exposure of LNCaP cells. However, in SS/DEHP-T, SM/DEHP-T, SS/MEHP-T, and SM/MEHP-T cells, significant increases (~20–25%) of GSH were observed compared to control cells (NT-C, SS-S, and SM-S cells).

GR activity of the cells, on the other hand, increased significantly with DEHP or MEHP treatment (1.7-fold and 1.8 fold, respectively). In SS/DEHP-T and SM/DEHP-T cells, GR activity increased further, reaching the levels of SS-S and SM-S cells. However, the increase observed in SS/MEHP-T and SM/MEHP-T cells was not found to be significantly different than that of MEHP-T cells.

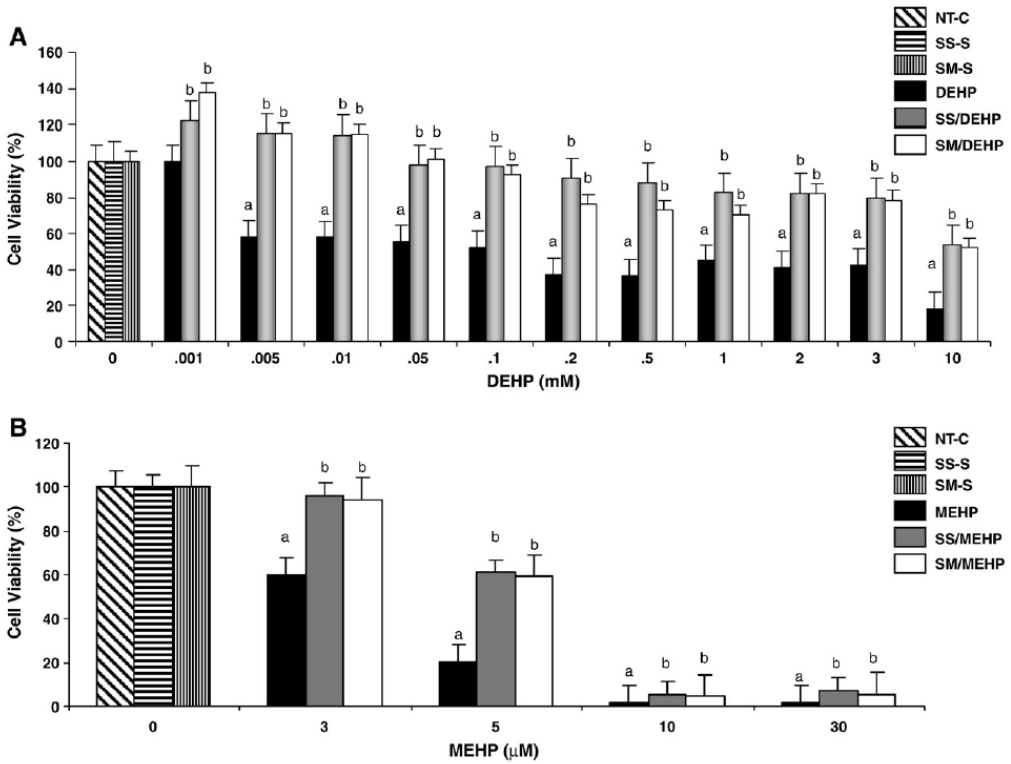

Fig. 1. Cell viability in phthalate-exposed LNCaP cell line, and protective effect of selenium supplementation. Cell viability was determined by the MTT assay and data were presented as relative to zero dose of DEHP or MEHP. Values are given as mean ± SEM of $n = 3$ experiments and triplicate measurements. (A) Cytotoxicity of various concentrations of DEHP on LNCaP cells cultured with or without selenium supplementation. NT-C, nontreated LNCaP cells cultured for 24 h; SS-S, LNCaP cells supplemented and cultured with 30 nM SS for 72 h; SM-S, LNCaP cells supplemented and cultured with 10 μM SM for 72 h; DEHP, LNCaP cells treated with various concentrations of DEHP for 24 h; SS/DEHP, SS-S cells cultured with various concentrations of DEHP for 24 h; SM/DEHP, SM-S cells cultured with various concentrations of DEHP for 24 h. [a] $P<0.05$ vs NT-C, [b] $P<0.05$ vs DEHP. (B) Cytotoxicity of various concentrations of MEHP exposure on LNCaP cells cultured with or without selenium supplementation. MEHP, LNCaP cells cultured with various concentrations of MEHP for 24 h; SS/MEHP, SS-S cells cultured with various concentrations of MEHP for 24 h; SM/MEHP, SM-S cells cultured with various concentrations of MEHP for 24 h. [a] $P<0.05$ vs NT-C, [b] $P<0.05$ vs MEHP.

DEHP and MEHP increased DNA damage in comet assay and selenium supplementation was partially protective

Table 2 summarizes the DNA damage produced by 3 mM DEHP and 3 μM MEHP in LNCaP cells with or without Se supplementation. To quantify the induced DNA damage, the tail moment which reflects the percentage of DNA in the tail of the comet multiplied by the tail length was used. Furthermore, the percentage of DNA in the tail of the comet, the tail intensity, was given in order to visualize the migration pattern of the DNA. Fig. 2 illustrates the examples of comet images of the treatment groups used in the study.

Both DEHP and MEHP produced significant DNA damage as evidenced by increased tail % intensity (~2.9-fold and ~3.2-fold, respectively), and tail moment (~2.4-fold and ~2.6-fold, respectively)

Table 1
Enzymatic and nonenzymatic antioxidant status in DEHP- or MEHP-treated LNCaP cells and effects of selenium supplementation

Treatment groups	TrxR (U/mg protein)	GPx1 (U/mg protein)	GST (nmol/min/mg protein)	GR (U/mg protein)	Total GSH (pmol/mg protein)
NT-C	25 ± 0.003^{a}	9.077 ± 1.194^{a}	58 ± 0.011^{a}	10 ± 0.001^{a}	130.592 ± 5.180^{a}
SS-S	81 ± 0.005^{b}	18.700 ± 3.270^{bd}	53 ± 0.003^{a}	17 ± 0.003^{bc}	132.176 ± 1.946^{a}
SM-S	78 ± 0.001^{b}	25.714 ± 1.386^{b}	54 ± 0.005^{a}	24 ± 0.002^{b}	133.507 ± 4.105^{a}
DEHP-T	19 ± 0.003^{a}	2.425 ± 0.150^{c}	65 ± 0.007^{a}	17 ± 0.001^{c}	128.450 ± 5.732^{a}
SS/DEHP-T	64 ± 0.007^{b}	10.131 ± 0.578^{ae}	58 ± 0.005^{a}	26 ± 0.003^{b}	157.863 ± 6.556^{b}
SM/DEHP-T	43 ± 0.001^{c}	15.905 ± 2.048^{de}	58 ± 0.004^{a}	25 ± 0.002^{b}	164.480 ± 2.953^{b}
MEHP-T	35 ± 0.004^{d}	1.908 ± 0.291^{c}	49 ± 0.006^{a}	18 ± 0.002^{bc}	125.710 ± 4.069^{a}
SS/MEHP-T	72 ± 0.006^{b}	3.467 ± 0.182^{f}	50 ± 0.004^{a}	21 ± 0.003^{bc}	163.198 ± 2.848^{b}
SM/MEHP-T	62 ± 0.007^{bc}	4.789 ± 0.464^{f}	46 ± 0.002^{a}	26 ± 0.003^{bc}	166.679 ± 9.292^{b}

Values are given as mean ± SEM of $n = 3$ experiments and triplicate measurements. Means within each row that do not share same letters (superscripts) are significantly different from each other ($P<0.05$). Measurements were performed in the following treatment groups of cells: NT-C, nontreated LNCaP cells cultured for 24 h; SS-S, LNCaP cells supplemented and cultured with 30 nM SS for 72 h; SM-S, LNCaP cells supplemented and cultured with 10 μM SM for 72 h; DEHP-T, LNCaP cells cultured with 3 mM DEHP for 24 h; SS/DEHP-T, SS-S cells cultured with 3 mM DEHP for 24 h; SM/DEHP-T, SM-S cells cultured with 3 mM DEHP for 24 h; MEHP-T, LNCaP cells cultured with 3 μM MEHP for 24 h; SS/MEHP-T, SS-S cells cultured with 3 μM MEHP for 24 h; SM/MEHP-T, SM-S cells cultured with 3 μM MEHP for 24 h.

Table 2
Results of alkaline comet assay in DEHP- or MEHP-treated LNCaP cells and effects of selenium supplementation

Treatment groups	Tail intensity (%)	Tail moment (arbitrary units)
NT-C	3.77 ± 0.29^{af}	1.53 ± 0.13^{ac}
SS-S	4.22 ± 0.028^{ac}	1.44 ± 0.032^{ac}
SM-S	3.57 ± 0.147^{ad}	1.38 ± 0.025^{a}
DEHP-T	10.81 ± 0.147^{be}	3.64 ± 0.656^{bd}
SS/DEHP-T	7.14 ± 0.077^{c}	2.51 ± 0.162^{abd}
SM/DEHP-T	6.85 ± 0.078^{cd}	2.98 ± 0.046^{abd}
MEHP-T	12.11 ± 0.308^{e}	3.91 ± 0.095^{b}
SS/MEHP-T	8.34 ± 1.487^{bcf}	2.96 ± 0.528^{bcd}
SM/MEHP-T	7.40 ± 0.368^{bc}	2.58 ± 0.184^{d}

DNA damage was measured as tail % intensity and tail moment. Values are given as mean ± SEM of $n = 2$ experiments and triplicate measurements. Means within each row that do not share same letters (superscripts) *are* significantly different *from* each other ($P<0.05$). Measurements were performed in the following treatment groups of cells: NT-C, nontreated LNCaP cells cultured for 24 h; SS-S, LNCaP cells supplemented and cultured with 30 nM SS for 72 h; SM-S, LNCaP cells supplemented and cultured with 10 μM SM for 72 h; DEHP-T, LNCaP cells cultured with 3 mM DEHP for 24 h; SS/DEHP-T, SS-S cells cultured with 3 mM DEHP for 24 h; SM/DEHP-T, SM-S cells cultured with 3 mM DEHP for 24 h; MEHP-T, LNCaP cells cultured with 3 μM MEHP for 24 h; SS/MEHP-T, SS-S cells cultured with 3 μM MEHP for 24 h; SM/MEHP-T, SM-S cells cultured with 3 μM MEHP for 24 h.

compared to nontreated LNCaP cells. The overall difference between the DNA damaging effects of the parent compound and the metabolite was insignificant.

Se supplementation itself did not cause any alteration in the steady-state levels of the biomarkers of DNA damage in LNCaP cells, whereas the presence of Se either in SS (30 nM) or SM (10 μM) form reduced the genotoxic effects of DEHP and MEHP as evidenced by significant (≥30%) decreases in tail % intensity. These results thus indicated that the Se with the doses and forms used in this study was not genotoxic, but showed antigenotoxic activity against the genotoxicity of DEHP and MEHP. However, the protective effect of Se with the doses used in this study was not complete. Tail intensity remained ~90 and ~80% higher than that of NT-C in SS/DEHP-T and SM/DEHP-T cells, respectively. Similarly, in SS/MEHP-T and SM/MEHP-T cells, tail intensities were still ~95 and 120% high compared to NT-C cells.

On the other hand, the extent of tail moment increase induced by DEHP was reduced ~30% with SS and ~18% with SM supplementations, and the tail moment induced by MEHP was reduced ~24% with SS supplementation; however, none of these were statistically significant. Only SM supplementation provided a significant (~34%) reduction in the tail moment induced by MEHP. But again, tail moments remained ~64 and ~95% higher than that of NT-C in SS/DEHP-T and SM/DEHP-T cells, respectively; similarly in SS/MEHP-T and SM/MEHP-T cells, tail moments were still ~94 and 69% high compared to NT-C cells. In all cases, protective effects of SS and SM were not significantly different than each other.

Discussion

Recent studies show that phthalates produce free radicals by several pathways including the activation of PPARα [28], and several data suggest that oxidative stress and mitochondrial dysfunction in germ cells may contribute to phthalate-induced disruption of spermatogenesis. Induction of oxidative stress by DEHP was reported as measured by increases in ROS in subsequently isolated rat spermatocytes [29]. MEHP was reported to increase peroxiredoxin 3 and cyclooxygenase-2 levels in germ cells, indicating the disruption of cellular redox mechanisms in spermatocytes [30]. Thus, at least one of the mechanisms underlying the reproductive toxicity of DEHP might be its ability to increase generation of ROS and/or to cause alterations on intracellular enzymatic and nonenzymatic antioxidants. On the other hand, a shift in the prooxidant–antioxidant balance within the prostate has been proposed as a factor that contributes to prostate carcinogenesis [7].

The studies described herein, therefore, investigated the effects of DEHP and its principle hydrolysis product, MEHP, on the survival, antioxidant status, and DNA damage of LNCaP human prostate cancer cells, and examined the possible protective effects of Se supplementation. The overall results showed that both DEHP and MEHP were cytotoxic in different dose ranges, impaired the antioxidant status in LNCaP cells, and caused oxidative DNA damage as evidenced by increase of tail % intensity and tail moment in alkaline comet assays. Supplementation with Se in the form of SS or SM was protective against cytotoxicity, restored the antioxidant status, and reduced the genotoxicity of DEHP and MEHP, suggesting that an oxidative stress process and/or an effect on the expression of antioxidant enzymes might be the underlying mechanisms of the cytotoxicity and genotoxicity produced by these phthalate derivatives in LNCaP cells.

Significant cytotoxicity was observed with 5 μM and higher concentrations of DEHP. However, in accordance with earlier reports, the cytotoxic potency of MEHP was much higher than the parent compound. Se supplementation was effective in protecting the viability of LNCaP cells exposed to various doses of DEHP, and MEHP. The protective effect of 30 nM SS supplementation on cell survival was as the same level as the protection provided by 10 μM SM. Thus, more SM was required to achieve a similar effect as that obtained with the more bioavailable form of Se, selenite. The effects of SS and SM on the biomarkers of cellular antioxidant defense and DNA damage in comet assay were also in the same range, except for a higher elevation in TrxR activity of MEHP-exposed cells only by SS supplementation. The doses of Se in the form of SS and SM used in this study were in the same range as those of SS and SM that were shown previously with the same properties for several other cell types [31,32]. SS is commonly used for cell culture and animal studies, and SM is the most common form of Se obtained from the diet. SM is converted to hydrogen selenide (H_2Se) through transulfuration and β-lyase cleavage, whereas SS interacts with GSH to form GSSeSG which is subsequently reduced to H_2Se. H_2Se derived via both pathways can be converted to selenophosphate which is then used in the synthesis of selenoproteins, or methylated into active metabolites, such as methylselenol. This difference in Se metabolism is likely to account for the greater efficiency of SS over SM, as has been reported for a variety of cell types [33].

The induction of GPx1 activity we observed in this study with Se supplementation was previously demonstrated in several tissues, and reported as being due to enhanced translation and not transcription of the enzyme [34]. The decreasing effect of DEHP and MEHP we observed on GPx1 activity was completely restored by Se supplementation to the steady-state level (that of NT-C) or even higher. However, GPx1 activity remained lower than those of SS-S and SM-S cells, indicating that both DEHP and MEHP interfered with the upregulation of GPx1 by Se. GPx1 is known to play an important role in the defense mechanisms of mammals against oxidative damage by catalyzing the reduction of H_2O_2 and a large variety of hydroperoxides with GSH as the hydrogen donor. Increasing numbers of reports show that overexpression of GPx1 is associated with a wide range of effects, including the prevention of apoptosis, the protection against toxicity, and the reduction of DNA damage [35], implying that GPx1 is a major antioxidant enzyme that protects cells against lethal oxidative stress. Moreover, accumulating data have implicated the GPx1 as a determinant of cancer risk and a mediator of the chemopreventive properties of Se [35].

Taking into account the critical role of GPx1 activity and expression, the reducing effect we observed in this study with DEHP and MEHP can be considered as having critical importance in the cellular redox status in prostate. Significant induction of GR activity by phthalate treatment supports further the production of oxidative stress, because GR, the important cellular antioxidant in maintaining high levels of reduced GSH in cells, is inducible on oxidative stress. On

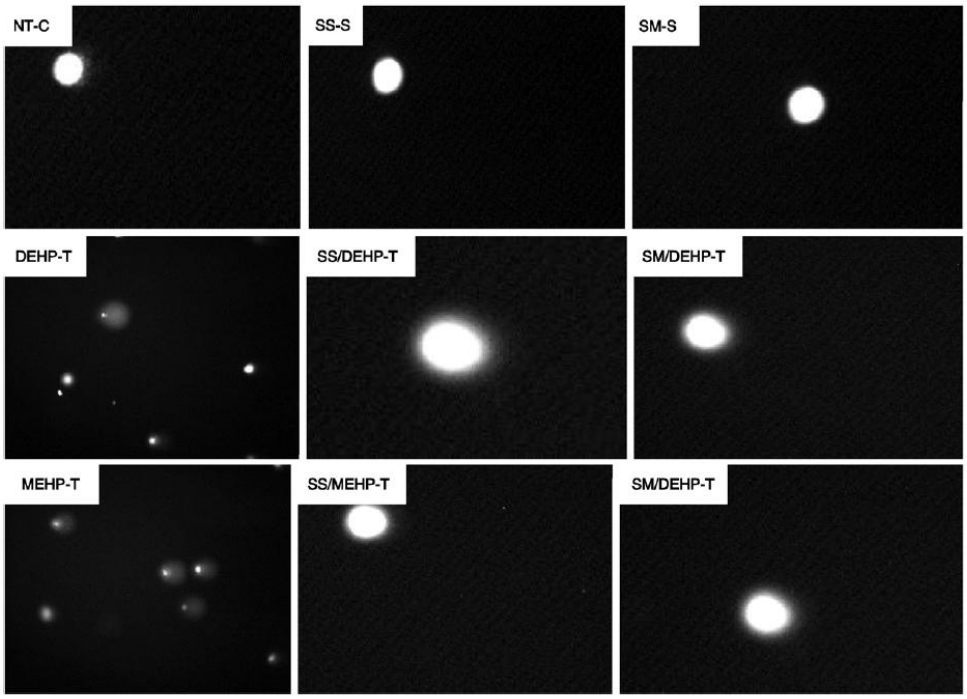

Fig. 2. Typical images of comets in DEHP- or MEHP-treated LNCaP cells and effects of selenium supplementation. Measurements were performed in the following treatment groups of cells: NT-C, nontreated LNCaP cells cultured for 24 h; SS-S, LNCaP cells supplemented and cultured with 30 nM SS for 72 h; SM-S, LNCaP cells supplemented and cultured with 10 μM SM for 72 h; DEHP-T, LNCaP cells cultured with 3 mM DEHP for 24 h; SS/DEHP-T, SS-S cells cultured with 3 mM DEHP for 24 h; SM/DEHP-T, SM-S cells cultured with 3 mM DEHP for 24 h; MEHP-T, LNCaP cells cultured with 3 μM MEHP for 24 h; SS/MEHP-T, SS-S cells cultured with 3 μM MEHP for 24 h; SM/MEHP-T, SM-S cells cultured with 3 μM MEHP for 24 h.

the other hand, GSTs are the GSH-related enzyme families that are capable of detoxifying genotoxic electrophilic compounds by catalyzing their conjugation to GSH [36]. However, except for a ~20–25% increase of total GSH in cells concomitantly exposed to DEHP or MEHP and Se, there was no phthalate treatment-related difference in GSH content, and none of the treatments used in this investigation caused a change in GST activity.

TrxR, like GPx1, is a Se-containing redox enzyme and together with thioredoxin (Trx) and NADPH comprises an important defense system against oxidative stress [37]. This so-called Trx system is also involved in many other biological processes, such as DNA repair, apoptosis, and regulation of several transcription factors [38]. Mammalian TrxRs are complex selenoenzymes that reduce Trx and detoxify hydroperoxides [38]. This reduction may be direct or by regeneration of cellular antioxidants [39]. Available data also indicate that cytosolic TrxR is regulated by the redox state of the cell; in fact it was shown that the addition of H_2O_2 led to increased TrxR activity in a small-cell lung carcinoma cell line [40]. Our findings showing an increase in TrxR activity by MEHP exposure, but no effect with DEHP, suggested that the responsible agent for changing the redox state of the cells was mainly MEHP. These results further suggested that GPx1 and TrxR were modulated differently in phthalate-exposed LNCaP cells.

The results of the comet assay of the present study clearly showed the genotoxic potential of DEHP and MEHP in LNCaP cells. Similar results have been demonstrated by others in different tissues and with various genotoxicity assays. Anderson et al. [17] observed genotoxic effects of DEHP and MEHP in human leucocytes and lymphocytes using the alkaline comet assay. Kleinsasser et al. [18] reported a dose-dependent enhancement of DNA migration by MEHP both in human mucosal cells and in lymphocytes. Hauser et al. [41] showed a relationship between urinary concentrations of phthalate metabolites, including MEHP, and sperm DNA damage in men. Thus, genotoxicity might have a contributory role on the effects of these compounds which have been known as nongenotoxic rodent carcinogens [10].

Our data demonstrating that Se supplementation was effective in reducing the genotoxicity of the DEHP and MEHP are in accordance with various previous reports in which the protective effect of Se was evaluated with various measurement methods including the comet assay. SS was significantly preventive against UV-mediated DNA damage in human skin fibroblasts [42]; SS and SM protected kerotinocytes from UV-induced DNA damage [43]; the extent of DNA damage in prostate cells and in peripheral blood lymphocytes (PBLs) was lower in Se-supplemented elderly dogs than the controls [44]. The latter investigators also observed a U-shaped dose–response relationship between Se status and extent of DNA damage within the aging prostate, suggesting that not all men will necessarily benefit by increasing their daily Se intake [45]. In fact, it is important to recognize the fact that Se is bimodal in nature whereby its beneficial properties occur in a fairly narrow range of daily intake. At low concentrations, Se compounds are antigenotoxic and anticarcinogenic, whereas at high concentrations, they are mutagenic, toxic, and possibly carcinogenic [46]. At moderate, supranutritional doses, Se compounds inhibit cell growth and posses a prooxidant activity, generating superoxide [47]. The prooxidant activity of Se seems to have both harmful and beneficial roles [48], and it appears that Se status has multilayered effects on both DNA integrity involving

antioxidative defenses, oxidative stress, and signaling pathways, and on the integrity and function of DNA repair proteins involving oxidation of SH groups and zinc release from zinc fingers [49]. On the one hand, there are data indicating that higher doses of some Se compounds including SS have the potential to induce DNA damage [46,50]. On the other hand, as already noted above, SS and/or SM were protective against UV-mediated DNA damage by enhancing DNA repair protein complexes [42,51], and that enhancement was suggested to be a mechanism of the chemopreventive effects of Se [52]. Recently, it was also reported that bleomycin-induced DNA damage in PBLs was repaired better in the presence of SM [53]. Taken together, it seems that many factors contribute to the great variety of results, mainly the chemical form and the concentration used, but also the exposure time, the treatment conditions, the cell type, and the target tissue [54]. In this regard, it appears that the doses and the chemical forms of Se we used in this study were appropriate, did not exert any genotoxicity in LNCaP cells, but provided protection against the genotoxic effects of DEHP and MEHP on LNCaP cells at doses used within the study. Bioinformatics and comparative phylogenetics analyses recently described 25 different human proteins containing selenocysteine [55], two of which, Sep15 and Selenoprotein P, are implicated in prostate cancer development and prevention [56,57]. Thus, along with GPxs and TrxRs which provide the first line of antioxidant defense against genotoxic damage, Se induces expression of other selenoproteins which may involve DNA synthesis, repair, or damage. Although the mechanism is not fully elucidated, the Trx system and thus TrxR is considered to be the main selenoenzyme involved in DNA repair. TrxR reduces many critical proteins and thus participates in DNA synthesis and repair, or redox signaling by hydrogen peroxide [58]. Trx-dependent redox regulation of p53 couples oxidative stress response and p53-dependent DNA repair and apoptosis. Fischer et al. [4] have shown that SM preferentially induced the DNA repair branch of the p53 pathway. However our data are too limited to make a conclusion in this respect, and more detailed studies would be needed including different dose levels and exposure times for both Se compounds and phthalates tested.

In conclusion, our data demonstrating the DNA-damaging effects of DEHP and MEHP in LNCaP cells and the disturbances they cause in the antioxidant status, particularly their reducing effect on GPx1 activity, are of importance with regard to the inevitable exposures to the phthalates and the high prevalence of prostate cancer. The effect of the Se supplementation we observed in this study is also in accordance with its protective effect against prostate cancer, its antioxidant properties, and upregulating effects on GPx1, TrxR, and DNA repair enzymes [4]. On the other hand, human serum typically contains ~1–2 μM Se, and Se concentration in specific organs can even be considerably less, particularly in prostate tissue. Culturing the cells in 10% serum as in the case of the presented studies is comparable to Se deficiency in people. Accumulating data have now implicated that both Se status and genetics, namely the genetic variants of GPx1, are associated with cancer risk for several types of malignancies [33,35]. Considering GPx1 activity reduction and DNA-damaging effects of DEHP and MEHP observed in the studies described herein, it can be concluded that even mildly Se-deficient individuals may be more susceptible to the adverse effects of these compounds. Therefore, the results presented herein emphasize once more the importance of Se status as a public health concern, and its importance with respect to the high probability of phthalate exposures and their toxic effects.

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Free Radical Research

Low doses of selenium specifically stimulates the repair of oxidative DNA damage in LNCaP prostate cancer cells

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Low doses of selenium specifically stimulates the repair of oxidative DNA damage in LNCaP prostate cancer cells

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Abstract

Epidemiological studies have demonstrated an inverse relationship between selenium (Se) intake and cancer incidence and/or mortality. However, the molecular mechanisms underlying the cancer chemopreventive activity of Se compounds remain largely unknown. The objective of this study was to investigate the effect of low doses of Se on the stimulation of DNA repair systems in response to 4 different qualities of DNA damage. P53-proficient LNCaP human prostate adenocarcinoma cells were grown either untreated or in the presence of low concentrations of Se compounds (30nM sodium selenite, or 10 µM selenomethionine) and exposed to UVA, H_2O_2, methylmethane sulfonate or UVC. Cell viability as well as DNA damage induction and repair were evaluated by the alkaline Comet assay. Overall, Se was shown to be a very potent protector against cell toxicity and genotoxicity induced by oxidative stress. Furthermore, Se-treated cells exhibited increased oxidative DNA repair activity, indicating a novel mechanism of Se action. Therefore, the benefits of Se could be explained by a combination of antioxidant activity and the enhancement of oxidative DNA repair capacity.

Introduction

Selenium (Se) is an essential trace element of fundamental importance to human health. It is present in the diet mostly in organic forms [1]. However, both organic and inorganic forms of Se appear to be utilized with similar efficacy in the body to produce selenoproteins [2], although they enter Se metabolic pathways at different points, depending on their chemical forms [3]. Se is bimodal in nature and its beneficial properties are apparent in a fairly narrow range of daily intake [4]. Se-containing compounds are involved in fundamental biological processes ranging from apoptosis to immunity [5], including cellular antioxidant defence as well as the protection and repair of DNA [6]. Selenoproteins are collectively essential for life and several of them, such as members of the thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) families, function as antioxidant enzymes [7]. However, the physiological roles of most of the other selenoproteins have not yet been fully elucidated [8].

Redox regulation has emerged as an essential process of many pathways in cell biology [9]. However, disruption of the intracellular redox balance leads to a state of oxidative stress, during which proteins, nucleic acids, lipids, and other macromolecules can accumulate damage. This can be accelerated by low dietary Se intake, resulting in the organism being prone to oxidative stress-related conditions, reduced fertility and immunity, and to increased risk of cancer [10]. In addition, Se supplementation of a population with low basal blood Se levels may have decreased the incidence of prostate cancer [11,12]. Several mechanisms of action have been proposed to account for this effect, including the stimulation of apoptosis [11,12], the induction of cell-cycle arrest[13], activation of the p53 tumor-suppressor protein [14], induction of DNA repair genes, enhancement of immune functions, and influencing estrogen- and androgen-receptor expression [15].

DNA damage caused by reactive oxygen species (ROS) includes a variety of lesions, ranging from base modification, to DNA breaks and DNA-protein cross-links. More than 60 different ROS-induced base lesions have been identified, the best characterized being 8-oxo-

7,8-dihydroguanine (8-oxoGua). If not repaired, this oxidized guanine results in GC to TA mutations [16]. 8-oxoGua is repaired *via* the base excision repair (BER) process initiated by a specific DNA glycosylase and an AP endonuclease. The actions of these two enzymes result in the generation of a single strand break (SSB) that requires further processing before gap filling and ligation. Interestingly, BER enzymes are sensitive to the redox status of cells [17,18].

Se may play a protective role in carcinogenesis by both decreasing the levels of DNA damage and modulating DNA repair activities. Thus, we designed the present study to investigate the effect of low concentrations of distinct selenocompounds on the cytotoxic and genotoxic properties of 4 different DNA damaging agents, as well as on the efficiency of DNA repair systems. The most robust results of the NPC Trial came from the analyses of the complete data for prostate cancer incidence. It showed that dietary supplementation with 200 µg/day of yeast-enriched selenium resulted in a 63% reduction in prostate cancer risk [19]. Moreover, because dogs and humans are the only two species in which prostate cancer occurs spontaneously with appreciable frequency, Waters et al. examined the effects of dietary selenium supplementation on DNA damage in elderly beagle dogs and found efficient protection [20]. Se-mediated protection of the prostate against cancer has been well-documented [21]. For these reason, we focus our work on cultured prostate cells, in particular LNCaP cell line. The proliferation of LNCaP cells is androgen-dependent and they are wild-type for gene suppressor p53. Therefore, in the present work LNCaP cells were used as an *in vitro* tool for assessing the protection afforded by Se against genotoxicity of several stress agents [22].

We used two different selenocompounds, sodium selenite (SS) an inorganic Se-derived and selenomethionine (SM) an organic one. These compounds are the two most common selenocompounds used in several epidemiological studies and present a different metabolism in cells.

Methods

Chemicals

Sodium selenite (SS), selenomethionine (SM), Dulbecco's Phosphate Buffered Saline (PBS) with $MgCl_2$ and $CaCl_2$ (PBS Ca^{2+} Mg^{2+}), MTT 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), crystal violet, bovine serum albumin (BSA), nuclease P1, DNAse II, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), Cell Lytic M cell lysis agent, protease inhibitor cocktail, and colorimetric assay kit for thioredoxin reductase (TrxR), were obtained from Sigma-Aldrich (St Quentin-Fallavier, France). The protein assay kit was from Uptima Interchim (Montlucon, France). NaOH was purchased from Carlo Erba (Rodano, Italy). RPMI 1640 medium and fetal calf serum (FCS) were from GIBCO (Courbevoie, France). Penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Microtitration plates were purchased from Nunc (Dutscher, Brimath, France). Formamidopyrimidine DNA glycosylase (Fpg), T4 endonuclease V (T4 Endo V) and Endonuclease III (Endo III) were Trevigen (Interchim, Montluçon, France). Phosphodiesterase II, phosphodiesterase I, and alkaline phosphatase were obtained from Roche Molecular Biochemicals (Indianapolis, USA).

Cell culture

LNCaP cells, which are wild type for both the p53 and Rb genes, were cultured in RPMI 1640 medium with 10% FCS and 1% penicillin/streptomycin in culture flasks at 37°C in 5% CO_2. For Se treatment, the medium was supplemented with either 30nM SS or 10 µM SM for 72 h. A no treatment control (NT) was included in each experiment.

Cytotoxicity assay

LNCaP cells were plated in 35mm plates or in 96-well microtitration plates (in 2 mL or 200 µL of cell culture medium, respectively) and were incubated at 37°C for 72h, in the presence of SS and SM.

Four different DNA damaging treatments were tested: UVA and UVC radiation; hydrogen peroxide (H_2O_2, added to the medium for 30 min on ice), and methylmethane sulfonate (MMS, added to the medium for 15 min at 37°C). MMS and H_2O_2 were diluted in PBS $Ca^{2+}Mg^{2+}$ whereas control cells were treated with PBS Ca^2 Mg^{2+} alone.

Cell viability was determined by the MTT assay 24 h hours after exposure to the treatment [22].

Clonogenic cell survival assay

LNCaP cells were plated in 35 mm plates (10.000 cell/ml) and incubated at 37°C for 72 h with SS and SM. The cells were then stressed as described for the cell viability assay and immediately re-plated in a 60 mm plates at a density of 50 cell/mL. The culture medium was changed every 3 days, and at the 12^{th} day, crystal violet 0.5% in H_2O/MeOH (1:1) was added to each plate. The plates were rinsed with water, dried and the colonies were counted.

Determination of selenoenzyme activities

Cells were removed by trypsinization and were lysed using CelLytic™ M Cell Lysis agent (Sigma) with a protease inhibitor cocktail, and then centrifuged at 1600 x g at 4°C for 10 min. After a second centrifugation at 4800 x *g* at 4°C for 20 min, enzyme activities were measured in the supernatant.

Cytosolic GPx (GPx1) activity was measured in a coupled reaction with glutathione reductase as described by Flohé et al.[23]. Cytosolic TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit (Sigma), as described

previously[24]. The protein content of the samples was determined by bicinchoninic acid assay (BCA) using a protein assay kit (Pierce) [25].

Alkaline single-cell gel electrophoresis (Comet Assay): Measure of DNA damage

To evaluate the extent of DNA damage by the Comet assay, LNCaP cells were pre-treated with 30 nM SS or 10 µM SM for 72 h at 37°C and exposed to 50 J/cm² UVA, 10 J/m² UVC, 200 µM H_2O_2 or 500 µM MMS. Control conditions were NT, SS and SM cells sham-treated with PBS. Immediately after treatment, cells were collected counted and then suspended in freezing medium (20% FCS and 10% DMSO) at a density of $1x10^6$ cell/mL, gently frozen to -80°C and stored in liquid nitrogen until use.

Frozen pellets of LNCaP cells (NT, SS, SM treated and the controls) were centrifuged at 300 x *g* for 5 min at 4°C. The cell suspension was added to low melting point-agarose at 37°C and 100 µL of the mixture was placed on a microscope slide coated with one dried layer of 1% normal agarose in PBS. After gelling on ice, the slides were immersed overnight in cold lysis buffer (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris, 1% N-lauroylsarcosine sodium salt, 1% Triton X-100, 10% DMSO, pH 10) at 4°C in the dark. Slides were washed 3 times for 5 min with reaction buffer (40 mM HEPES, 0.1M KCl, 0.5mM Na_2-EDTA, 0.2 mg/mL BSA). Digestion with lesion-specific endonucleases (100 µL per slide) was performed for 30 min at 37°C. Cells on control slides were incubated only with enzyme buffer. After incubation, slides were immersed in pre-cooled electrophoresis buffer (1 mM Na_2-EDTA and 300 mM NaOH) for 30 min in order to allow the unwinding of the DNA and then electrophoresis was applied at 25 V/300 mA for 30 min. After electrophoresis, slides were rinsed three times for 5 min with neutralization buffer (0.4 M Tris-HCl, pH 7.4), stained with ethidium bromide (100 µL per slide, 2.5 µg/mL) and covered with a cover slip prior to analysis.

A fluorescence microscope (Carl Zeiss, Germany) connected to a charge-coupled device (CCD) camera and a computer-based analysis system (Comet assay IV software, Perceptive Instruments Ltd) was used to determine the extent of DNA damage after electrophoresis of DNA fragments in the agarose gel. The extent of damage was evaluated as the average of the triplicate values of the tail DNA intensity (% of DNA in the tail of the comet).

Assessment of cellular DNA repair capacity in protein extracts

Substrate preparation

Cells were exposed to an oxidative agent (Riboflavin 1µM + UVA 10 J/cm²) inducing 8oxoGua, an alkylating agent (MMS, 500µM) and a pyrimidine dimer-forming agent (UVC 10 J/m²), collected by trypsinization and suspended in freezing medium slowly frozen to -80°C and stored in liquid nitrogen.

Preparation of whole cell extracts

Liquid nitrogen-stored frozen aliquots of LNCaP cells (NT, SS, SM conditions) were centrifuged at 300 x *g*, 4°C for 5 min. A dry pellet was subsequently frozen in liquid nitrogen and resuspended in 33 µL of extraction buffer (45 mM HEPES, 0.4M KCl, 1 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol [DTT], 0.25% Triton-X 100, pH 7.8). The mixture was then vortexed for 30 sec, incubated for 5 min on ice and centrifuged at 14000 x g for 5 min at 4°C. The supernatant was then combined in a 1:5 ratio with reaction buffer. Extracts (50 µL) were placed on each slide covered with a cover glass and incubated for 30 min at 37°C. Samples digested with specific purified repair enzymes were used as positive controls while negative controls were incubated only with a 50 µL mix of extraction and reaction buffers. After digestion, the slides were transferred to an electrophoresis tank and processed using the Comet assay protocol described above.

High pressure liquid chromatography- tandem mass spectroscopy detection of 8-oxoGua

LNCaP cells (NT, SS and SM conditions) were irradiated in PBS in plastic petri dishes. DNA was extracted from cells using a sodium iodide (NaI) precipitation based chaotropic method [26], and solubilised in 50 µL of deferoxamine. DNA samples were hydrolysed by incubation with nuclease P1, phosphodiesterase II, DNase II, phosphodiesterase I and alkaline phosphatase [27]. Samples were then analysed by high performance liquid chromatography associated with tandem mass spectrometry (HPLC-MS/MS) with electrospray ionization. The apparatus consisted of SCIEX API 3000 electrospray triple quadrupolar spectrometer associated with a series 1100 Agilent chromatographic system. Quantification of DNA damage was performed in the multiple reactions monitoring mode as previously described [28].

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Statistical significances of differences among treatment groups were determined by Student's t-test. A p-value of <0.05 was considered as statistically significant.

Results

Selenoenzyme activities

The modulation of the activity of the GPx1 and TrxR selenoenzymes by low concentrations of Se (in the form of SS and SM) in LNCaP cells are summarized in Table 1. Se supplementation of LNCaP cells with either SS or SM significantly ($p < 0.05$) increased the activities of TrxR (~3-fold) and GPx1 (≥ 2-fold).

Low-dose of selenium supplementation increases resistance to UVA and H_2O_2-induced cytotoxicity

For all cytotoxicity assays, cells were pre-treated with SS or SM during 72 hours. After 3 days cells were treated with several DNA-damaging agents and 24 h after that we measured cell viability.

Moreover, to understand whether selenium supplementation was fundamental to prevent cellular death or it was able to ameliorate cell survival also after treatment we also carried out selenium post-treatment. That is, LNCaP cells were plated for 72 hours, then cells were stressed and immediately post-treated or not with SS and SM for 24h.

Figure 1A shows the % viability of the cells irradiated with increasing doses of UVA. Cells pre-treated with Se exhibited a better survival than controls after UVA irradiation ($p<0.01$). After exposure to 25 J/cm² of UVA, SS and SM pre-treated cells were fully viable whereas viability of NT cells was only 30%. In contrast, post-treatment with selenocompounds had no effect on cell survival.

Similar observations were made with H_2O_2 (Figure 1B). Pre-treatment with SS and SM resulted in greater cell survival when compared to NT ($p<0.05$). The LC_{50} of H_2O_2 in NT cells was 200 µM whereas SS and SM pre-treated cells had an LC_{50} of 5mM of H_2O_2.

The next type of stress was a monofunctional alkylating agent, namely MMS. Compared to the previous treatments, the MMS incubation did not highlight any effects of SS and SM treatment on cell survival (Figure 1C).

In contrast to oxidative stresses, there was not protective effect of SS and SM against cell death after exposure to UVC. UVC radiation cause only limited ROS formation and oxidative stress to cells (Figure 1D). On the other hand, the main lesions induced by UVC are bulky adducts (CPDs and (6-4)PP).

The next step was to confirm the Se-mediated modulation of the cellular response to stress by a clonogenic cell survival assay. This cell survival assay is based on the ability of a single cell to grow into a colony, and includes long term effects of exposure (12 days).

The clonogenic cell survival assay was carried following treatment with UVA or H_2O_2 for which selenium protection was shown in short-term survival. The results of all two treatments showed a significantly Se-protection of cellular response to oxidative stresses (Fig. 2).

Selenium protects specifically against oxidative DNA damage

The standard version of the Comet assay, when performed under alkaline conditions, provides data for evaluating initial DNA strand breaks and alkali-labile sites (ALS) induced by exposure to different types of genotoxicity-inducing stress. In order to obtain information on specific categories of DNA base lesions, several glycosylases were used to convert base lesions to strand breaks. These three glycosylases are Fpg, Endo III or T4 Endo V, which removed respectively oxidized purines, oxidized pyrimidines and cyclobutane pyrimidines dimers.

Fig. 3(A) shows an example of undamaged and damaged DNA visualized by the Comet assay. The presence of strand breaks into DNA makes it more flexible. The growing tail intensity is proportional to DNA strand breaks. The incubation with Fpg, or with another glycosylases, allows to excise the Fpg-sensitive sites or other enzyme-sensitive site and thus to convert an oxidized purine or another base damage into strand breaks which can be detected by the increase of tail intensity.

The tail intensities measured for controls (NT, SS and SM) were very small and were not significantly increased upon incubation with the three repair enzymes.

The four stress agents chosen produced significant levels of DNA breaks as evidenced by increase of tail intensity.

In Fig. 3(B) we can observe an increase of tail intensity of approximately 6-fold after exposure to 50 J/cm^2 of UVA and ~10-fold after treatment with 200 µM of H_2O_2 compared to control cells. Exposure to UVA and H_2O_2 produced significant increase in the level of oxidized bases as evidenced by increase in tail intensity after Fpg incubation. Treatment with either 30 nM SS or 10 µM SM reduced the levels of DNA strand breaks induced by UVA or H_2O_2 as evidenced by significant decreases in tail intensity.

The increase of strand breaks frequency was of ~4-fold after incubation with 500 µM of MMS (Fig. 3(C)). Exposure to the monofunctional alkylating agent MMS produced significant alkylated bases as evidenced by increase in tail intensity after Fpg and EndoIII digestion (~4.5- and ~2.7-fold respectively). In contrast to oxidative stress agents, no significant differences in tail intensity was observed after SS or SM treatments

The increase of strand breaks frequency was of ~4 fold after exposure to 10 J/m^2 of UVC (Fig. 3(D)) compared to control cells. This UVC dose corresponds to 80% of cell viability obtained by MTT assay.. UVC exposure produced cyclobutane pyrimidine dimer (excised by T4 EndoV) as evidenced by the 9-fold increase in tail intensity as compared to control. Same to MMS, no significant differences in tail intensity was observed after SS or SM treatments.

Formation of 8-oxoGua upon UVA irradiation

As a complementary approach to the assessment of oxidative DNA lesions by the Fpg/Comet assay, the formation of 8-oxoGua after exposure to different doses of UVA radiation was quantified by HPLC-MS/MS analysis in NT, SS and SM cells (Figure 4). SM but SS exposure significantly decreased the induction of 8-oxoGua as compared to NT at 200 J/cm^2 ($p<0.05$).

Selenium supplementation increases 8oxoGua excision capacity

To study the effect of Se supplementation on DNA repair, we used a Comet assay-based approach, which assessed the ability of the repair enzymes present in cellular extracts to incise the DNA substrate and cause single-strand breaks. The initial steps of the repair processes include the sensing of the DNA damage, followed by the excision of the damage, resulting in single-strand breaks. The increase in the tail intensity after incubation of the damaged DNA in the cell extract is an indication of enhanced repair capacity of the enzymes involved. The specificity of the assay was provided by the nature of the lesions present in the substrate. Using substrates with distinct classes of damage, both BER and nucleotide excision repair (NER) can be assessed.

DNA tail intensities for the oxidized substrate, measured following incubation with all three cell extracts (NT, SS, SM) were significantly higher compared to incubation in buffer alone ($p<0.05$) (Figure 5A). The respective ratios were of 1.3-, 2.0- and 1.9-fold. In addition, protein extracts from cells pre-treated with SS and SM exhibited a significantly higher excision potential (30.5 ± 4.3 and 29.9 ± 3.3 respectively) than the NT LNCaP cells (20.1 ± 3.3, $p<0.05$). As a positive control, we evaluated the Fpg sensitive-sites values (46.5 ± 4.0) and they were significantly higher than the three cell extracts values.

A second DNA substrate from MMS-treated cells was also used in the repair assay. Unlike the results obtained with UVA/Riboflavin damaged DNA substrate, there were no significant differences in repair capacities for the protein extracts obtained from the SS and SM treated cells as compared to the NT control cells (Figure 5B). It is interesting to note that the activities of the positive controls (Endo III and Fpg) were lower compared to those of cell extracts. A similar trend was obtained for UVC-irradiated substrates (Figure 5C). The DNA tail intensities (%) for all three cell extracts (NT, SS, SM) were significantly higher compared to the tail intensity of the substrate treated with the buffer. However, no differences were found among the activities of the extracts. The efficiency of T4 Endo V activity was 2.5-fold higher than the activity achieved using the cell extracts.

4. Discussion

In the present study, the protection offered by supplementation with Se, in both organic (SM) and inorganic (SS) forms, against several DNA damaging agents was evaluated in a human prostate cancer cell line. Four types of agents were chosen in order to generate different types of DNA lesions, the repair of which are carried out by different DNA repair systems. DNA damage induced by UVA is thought to occur *via* an indirect mechanism, involving the generation of ROS, which leads to the formation of 8-oxoGua [29]. H_2O_2 treatment induces several types of lesions in DNA, including single or double-strand breaks and a variety of oxidized purines and pyrimidines [30]. MMS is an alkylating agent and its exposure results the predominant adducts in double-stranded DNA being methylated purines [31], each of these kinds of lesions are repaired by the BER system. The primary DNA lesions induced by UVC light are cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP), both of which are repaired by NER [32].

The concentrations of SS and SM used in this study were similar as those shown previously by our group to protect several other cell types from DNA damage [22,33]. SS is commonly used for cell culture and animal studies, and SM is the most common organic form of Se obtained from the diet. The difference in entry points in Se metabolism is likely to account for the greater efficiency of SS over SM [34]. It should be emphasized that our results indicated that only the metabolized form (or forms) of Se were effective in providing protection as addition of SS or SM immediately after agent exposure had no effects on the studied endpoints. This immediate effect rules out the involvement of *de novo* synthesized Se-containing compounds in genoprotection.

The selenocompounds used in the study were not cytotoxic at the concentrations tested and were both able to enhance viability resulting from UVA and H_2O_2 exposure and afforded greater clonal survival. This finding is consistent with the observation discussed above of

significant increases in the activities of GPx1 and TrxR selenoproteins. The induction of GPx1 activity with Se supplementation was previously demonstrated in several tissues and cell lines, and was reported to be due to the enhanced translation, and not transcription [35]. However, the selenocompounds tested were not protective against MMS and UVC-induced cytotoxicity, indicating that Se supplementation supplied protection only against ROS-induced cytotoxicity.

The extent of DNA damage was also investigated by two complementary approaches: the Comet assay and HPLC-MS/MS assessment of 8-oxoGua levels. Although the Comet assay is sensitive and qualitative, it is not adequate to quantitatively assess specific base damage, which was obtained by HPLC-MS/MS analysis. We observed that Se can modulate the induction of damage after exposure to DNA damaging agents. This was demonstrated for the induction of strand breaks induced by UVA and H_2O_2, as well as 8-oxoGua resulting from UVA irradiation. The significant decrease of 8-oxoGua formation in Se-treated cells after UVA irradiation was confirmed only after HPLC-MS/MS analysis. These results are therefore consistent with the cell survival data indicating that Se compounds are efficient at protecting cells from oxidative damage but not from agents that induce other types of deleterious lesions.

The interesting cytotoxicity results have highlighted that a pre-treatment with SS or SM confers a significantly increase of cell survival in response to an oxidative stress. Even more interesting is the important decrease of DNA damage by selenium supplementation still after oxidative stress. We believe that the action of selenium is mediated by the activity of antioxidant enzymes leading to a reduction of ROS. More complex cellular signaling mechanisms and biological responses could also be involved. For this purpose, the second part of the work will be devoted to the effect of selenium pretreatment on DNA repair.

We also examined the effect of Se on DNA repair capacity using a modified-Comet assay to measure excision activity of the protein extracts from NT, SS and SM cells. Extracts

of cells pre-treated with Se recognized and removed greater amounts of oxidized DNA bases than the NT extracts. Moreover, a clear bias toward improved response afforded by Se was observed for oxidative stress. In contrast, the repair capacity of cell extracts (NT, SS, SM) on MMS- and UVC-treated substrates were similar.

The pathways involved in the repair of lesions produced by the three stress-inducers are quite different. Since UVC-induced photoproducts are repaired by the NER pathway, our results indicated that the pathways involved in NER were probably not affected by Se supplementation. However, selenium in the form of selenomethionine was shown to enhance NER as measured by host-cell reactivation in human fibroblasts [36].

The differential response in repair achieved by Se supplementation of the medium of cells from which extracts were prepared to repair UVA/Riboflavin and MMS treated substrates was likely due to effects on the different glycosylases involved in the detection and removal of the two types of DNA damage. The glycosylase involved in the first step of 8-oxoGua detection is hOGG1, while that which detects methylated purines are N-methylpurine-DNA glycosylase (MPG) and 3-methyladenine-DNA glycosylase (AAG). However, the predominant MMS-induced lesion is N^7MeG, which can be more genotoxic by conversion of the primary lesion to Fapy-N^7MeG, an adduct removable by OGG1 [31]. The variety of DNA damage caused by MMS treatment is likely to explain the higher excision activity of the tested cell extracts compared to that seen with Fpg and Endo III as extracts can support the excision of more kinds of lesions induced by MMS above that which can be recognized by the two individual enzymes.

The improved repair of oxidized lesions obtained by pre-treatement of cells with SS and SM is likely to be linked to the redox properties of enzymes whose levels are increased by Se supplementation. The AP site is the major product of OGG1 and its lyase activity is the rate-limiting. Human APE1 significantly stimulates OGG1 activity *in vitro* [37,38]. Moreover, APE1 is modulated post-transcriptionally *via* redox modification by thioredoxin (Trx). In

turn, the redox activity of Trx is maintained by the TrxR selenoprotein. Trx has a critical role in regulating cellular redox status and it modulates APE1 redox activity. Therefore, the translocation of Trx from cytoplasm to nucleus, which is stimulated by the different Se compounds, indicates that cellular redox regulation entails a complex cascade of events [37]. Moreover, hOGG1 possesses critical redox-sensitive residues and being in the reduced state is important for 8-oxoGua DNA glycosylase activity. In particular, molecular epidemiologic case-control studies indicated that a polymorphic variant of hOGG1 (serine to cysteine amino acid substitution at position 326) is associated with a higher risk of developing several types of cancer [38]. The hOGG1-Cys variant is particularly sensitive to the cellular redox status, for example, hOGG1-Cys enzymatic activity decreases after a pro-oxidant treatment [39]. These findings affirm the central importance of oxidative stress in carcinogenesis since it not only induces DNA damage but can also inhibit the cellular protection against such stress. The redox sensitivity of the hOGG1 pathway may explain how Se supplementation and its subsequent stimulation antioxidant defences contribute to the maintenance of a high hOGG1 activity explaining the basis of the enhanced excision activity achieved in Se-treated cell extracts for oxidative damage but not for agents whose exposure results in DNA alkylation.

The cellular redox status may also influence repair activity by modulating specific signal transduction pathways. In that respect, p53 appears as a potential target for Se-mediated protection. This may be important because p53 mediates cellular responses fundamental to carcinogenesis including cell cycle arrest, apoptosis and DNA repair. Seo et al. reported that p53 activity can be augmented by antioxidant mechanisms, thereby protecting cells from DNA damage. SM modulated p53 activity by causing redox regulation of key p53 cysteine residues (275/277) [18]. The resulting conformational shift leads to the induction of p53 DNA-binding activity and the stimulation of DNA repair [14,40]. Smith et al. showed that SS not only caused the reduction of cysteines in p53 as did SM, but also the phosphorylation of p53 at serines 20, 37, and 46 positions which are known to mediate apoptosis [41]. These results

contrast with our study as we have shown that SS protected cells against the oxidative stress. However, it should be emphasized that the concentration of SS used by Smith et al. was 300 fold higher than the present study. This difference reflects the well known biphasic effect of Se where it is toxic in high concentrations but not in physiological levels. Indeed, there is an intriguing U-shaped dose-response relationship between Se status and the extent of DNA damage within the prostate [44]. The same research group demonstrated that dietary Se supplementation decreased DNA damage and increased epithelial cell apoptosis within the aging canine prostate tissue [20]. These findings emphasizes that the baseline in nutrient status in the suboptimal range should be considered as a required entry criterion for prevention trials with Se in order to avoid the potential deleterious effects of over-supplementation. In October 2008, the large human prostate cancer prevention trial SELECT was stopped in its seventh year, and the results indicated that there were no beneficial effects of Se against cancer incidence [45]. However, this trial did not test different formulations or doses of Se and there has not been any information as to whether Se might be effective among those with the lowest baseline Se levels [42].

To conclude, our study established that supplementation of cells with low, physiologically relevant concentrations of Se protected LNCaP cells against the cytotoxic and genotoxic effects of oxidative stress by mechanisms likely including the enhanced repair of oxidative DNA lesions. These results confirm the data obtained from our previous studies in which we showed that Se in both SS and SM forms protected LNCaP cells and Leydig against phthalate-induced oxidative stress [33]. The absence of protection against other types of stress, either alkylating or radiating (UVC), emphasizes that SS and SM were only able to protect against oxidative stress and may only be regulators of intracellular redox status. Interestingly, SS was found to be 300 times more efficient than SM. However, another Se compound, diphenyl diselenide, was reported to exhibit protection against MMS and UVC treatment [47]. These observations indicate that the protective efficiency of Se derivatives strongly depends

on the chemical form and further studies should consider including other cell types and further work for better understanding of the regulation of the DNA repair by Se.

Declaration of interest

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Table 1. Antioxidant selenoenzyme activities in LNCaP cells and effects of Se supplementation.

	NT	SS	SM
TrxR (U/mg protein)	0.025±0.005	0.081±0.009*	0.078±0.002*
GPx1(U/mg protein)	9.0±2.1	18.7±5.6*	25.7±2.4*

* $p<0.05$ vs NT.

Figure legends

Fig. 1 Se supplementation increases resistance to oxidative stress.

Cell viability was determined by the MTT assay 24 hours after exposure and data were presented relative to the control cells. LNCaP cells were pre-treated or post-treated with 30 nM SS or 10 µM SM for 72 h or 24 h **(A)** Cells were irradiated with increasing doses of UVA. **(B)** Cells were incubated with several concentrations of H_2O_2 (30 min on ice). **(C)** Cells were incubated with several concentrations of MMS (15 min at 37°C). **(D)** Cells were irradiated with increasing doses of UVC. Values are given as mean ± SD of n=3 independent experiments and triplicate measurements, and we used for statistical significance using Student's t-test * $p<0.05$ and ** $p<0.01$ *vs* NT.

Fig. 2 Se supplementation increases long-term resistance to oxidative stress

Clonogenic cell survival assay (12 days) were performed in LNCaP cells pre-treated with SS or SM for 72 h, then irradiated with growing dose of (A) UVA or (B) incubated with several concentrations of H_2O_2 (30 min on ice) and re-plated in 100 mm petri dishes with 200 cells for 12 days. Values are given as mean ± SD of n=3 independent experiments and triplicate measurements, and we used for statistical significance using Student's t-test * $p < 0.05$ and ** $p<0.01$ *vs* NT.

Fig. 3 Selenium protects against oxidative DNA damage, assessed by alkaline comet assay after UVA, H_2O_2, MMS and UVC-treated LNCaP cells.

DNA damage was quantified as tail intensity (%). **(A)** Typical images of comets in UVA-treated LNCaP cells and effects of selenium supplementation are presented. LNCaP cell line was cultured with or without 30 nm SS or 10 µM SM for 72 h; each group was treated with a specific dose of **(B)** UVA (50 J/cm²), H_2O_2 (200 µM for 30 min on ice), **(C)** MMS (500 µM

for 15 min at 37°C) and **(D)** UVC (10 J/m²). Immediately after stress, cells were collected and comet assay was performed.

Values are given as mean ± SD of n=3 independent experiments and triplicate measurements. The mean of the tail intensity (n=3) was calculated and analyzed for statistical significance using Student's t-test. (* $p<0.05$ *vs* NT UVA and NT H_2O_2; $^{\Delta}$ $p<0.05$ *vs* NT UVA Fpg and NT H_2O_2 Fpg).

Fig. 4 Dose-response curve for the formation of 8-oxoGua after UVA irradiation detected by HPLC-MS/MS analysis.

Cells were pre-treated with SS or SM for 72 h and then irradiated with growing dose of UVA (0, 50, 100 and 200 J/cm^2). Immediately after irradiation cells were collected and DNA samples were extracted and hydrolyzed. HPLC coupled with tandem mass spectrometry was used to quantify 8-oxoGua lesion amount.

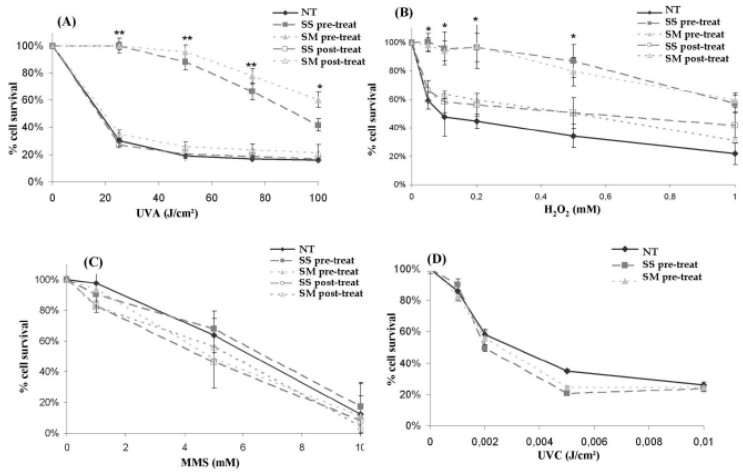
Values are given as mean of 8-oxoGua per 10^6 normal bases ± SD of n=3 independent experiments and triplicate measurements and analyzed for statistical significance using Student's t-test. * $p < 0.05$ *vs* NT

Fig. 5 Selenium supplementation increases 8oxoGua excision capacity of cell extracts .

Total protein cell extracts (NT, SS, and SM) were prepared and their excision capacities were tested into different DNA damaged substrate during 30 minutes at 37°C, using a modified version of comet assay. Three different DNA damaged substrate were used (A) 1µM Riboflavin + 10 J/cm^2 UVA, (B) 500 µM MMS, (C) 10 J/m^2 UVC. For each substrate we used an undamaged substrate as a control. Incubation with several repair enzymes was used as positive control (Fpg, Endo III, and T4 Endo V). Three biological replicates were tested in triplicate. The mean of % of excision of each cell extract (n=3) was calculated and statistical

significance was assessed using Student's t-test ($p<0.05$ vs buffer; * $p<0.05$ vs NT cell extract, # $p<0.05$ cell extract).

Abbreviations: **6-4 PP,** 6-4 photoproducts ; **8-oxoGua**, 7,8-dihydroguanine; **ALS**, alkali-labile sites; **AP**, apurinic sites; **APE1**, AP endonuclease 1; **BER**, base excision repair; **CPD,** cyclobutane pyrimidine dimer ; **DTT**, dithiothreitol; **Endo III**, endonuclease III; **Fpg**, formamidopyrimidine-DNA glycosylase; **GPx1**, glutathione peroxidase 1; **GR,** glutathione reductase; **H_2Se**, hydrogen selenide; **MMS**, methylmethane sulfonate; **MTT,** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **NER,** nucleotide excision repair; **hOGG1**, human 8-oxoguanine-DNA glycosylase; **ROS**, reactive oxygen species; **Se**, selenium; **Sec**, selenocysteine; **SM**, selenomethionine; **SS**, sodium selenite; **T4 Endo V**, T4 endonuclease V; **TrxR**, thioredoxin reductase;



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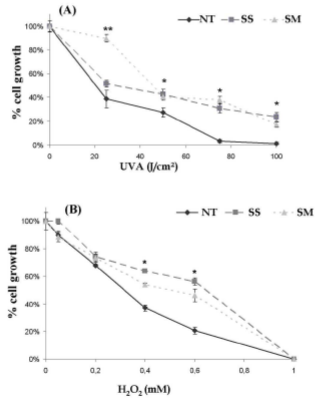

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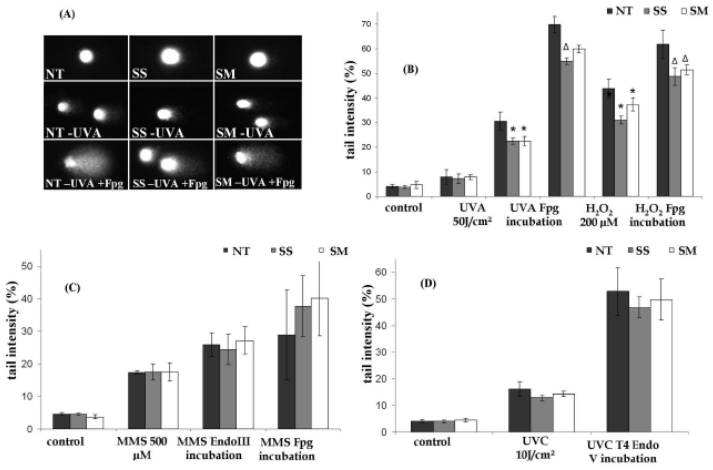

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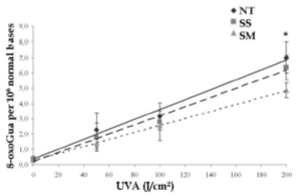

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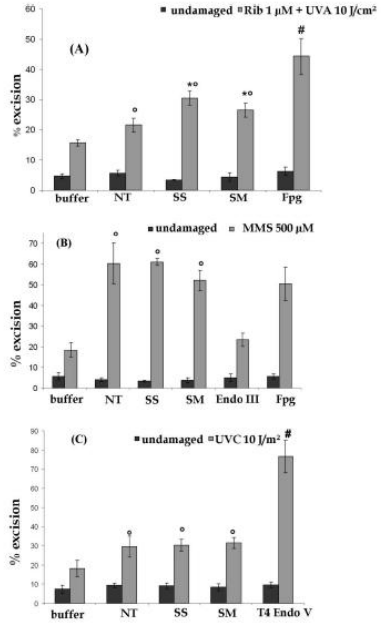

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Abstract

Selenium was recognized as an important micronutrient for both humans and animals. Several studies showed that increased selenium in the diet might be beneficial against liver, colon, pancreas and prostate cancer. The anticarcinogenic actions of Se occur at the systemic, cellular and nuclear level. These actions may also involve the immune system and thus cannot be interpreted by a single mechanism. Until now its mechanisms of action are not well understood. The objective of this study was to investigate the effect of selenocompounds at low doses on DNA repair capacity in the p53-proficient LNCaP prostate cancer cells. This work is divided into three parts. The first part of the work was devoted to study the effect of two selenocompounds (SS and SM) on the cytotoxic and genotoxic properties of various oxidative and non oxidative stresses. The results showed that low doses of Se pre-treatment stimulates selenoprotein synthesis, protects against toxicity and oxidative DNA damage induced by UVA or H_2O_2 but not by MMS or UVC. The second part of our investigation was devoted to the influence of selenium supplementation on DNA repair capacity. Our work clearly showed an increase in excision efficiency of the glycosylases activity that was not necessarily correlated with an increase of gene expression and/or protein levels. Finally, the third part of our work was devoted to the optimization of Host Cell Reactivation assay (HCR) to study the DNA repair capacity *in cellulo*, in order to target the partners involved in the signalling pathway affected by selenium supplementation. In conclusion, we could image that the mechanism of action of selenium is represented by a delicate balance between activation and repression of protein activity that induces conformational changes of several proteins more or less directly involved in DNA repair and progression of cell growth.

Key words: cancer prevention, DNA repair, OGG1, oxidative DNA damage, selenium

Résumé

Le sélénium est reconnu comme un micronutriment important pour l'homme et les animaux. Plusieurs études ont montré qu'une supplementation en sélénium dans le régime alimentaire pourrait être bénéfique contre les cancers du foie, du colon, du pancréas et de la prostate. Le mécanisme anti-carcinogène du sélénium se produit au niveau systémique, cellulaire et nucléaire. Ces processus peuvent également impliquer le système immunitaire et ne doivent pas être interprétés par un seul mécanisme. Jusqu'à présent son mécanisme d'action est encore inconnu. L'objectif de cette étude était d'étudier l'effet des composés du sélénium, à faibles concentrations, sur la capacité de réparation de l'ADN dans les cellules du cancer de la prostate LNCaP (p53 compétentes). Ce travail est divisé en trois parties. La première partie du travail a été consacrée à étudier l'effet des deux composés du sélénium (SS et SM) sur les propriétés cytotoxiques et génotoxiques de différents stress oxydatifs et non oxydatifs. Les résultats ont montré qu'un prétraitement avec une faible dose en Se stimulait la synthèse des sélénoprotéines, et protégeait contre la toxicité et les dommages oxydatifs à l'ADN induites par les UVA ou H_2O_2, mais pas par MMS ou UVC. La deuxième partie a été consacrée à l'influence de la supplementation en sélénium sur la capacité de réparation de l'ADN. Notre travail a clairement montré l'augmentation de l'efficacité d'excision de certaines glycosylases que n'est pas nécessairement corrélée à une augmentation de l'expression génique et /ou protéiques. Enfin, la troisième partie de notre travail a été dédiée à l'optimisation de la technique Host Cell Reactivation (HCR) qui nous a permis d'étudier la capacité de réparation de l'ADN *in cellulo*, afin de cibler les partenaires impliqués dans la voie de signalisation affectées par la supplémentation en sélénium. En conclusion, nous pourront penser que le mécanisme d'action du sélénium est représenté par un délicat équilibre entre l'activation et la répression de l'activité de certaines protéines qui induit des changements conformationnels plus ou moins directement impliqués dans la réparation de l'ADN et la progression de la croissance cellulaire.

Mots clés: sélénium, stress oxydant, dommage à l'ADN, réparation de l'ADN, OGG1,