

# Long-Term Selenium Supplementation in HaCaT Cells: Importance of Chemical Form for Antagonist (Protective Versus Toxic) Activities

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**Abstract** The beneficial effect of selenium (Se) on cancer is known to depend on the chemical form, the dose and the duration of the supplementation. The aim of this work was to explore long term antagonist (antioxidant versus toxic) effects of an inorganic (sodium selenite, Na<sub>2</sub>SeO<sub>3</sub>) and an organic (seleno-L-methionine, SeMet) forms in human immortalized keratinocytes HaCaT cells. HaCaT cells were supplemented with Na<sub>2</sub>SeO<sub>3</sub> or SeMet at micromolar concentrations for 144 h, followed or not by UVA radiation. Se absorption, effects of UVA radiation, cell morphology, antioxidant profile, cell cycle processing, DNA fragmentation, cell death triggered and caspase-3 activity were determined. At non-toxic doses (10 μM SeMet and 1 μM Na<sub>2</sub>SeO<sub>3</sub>), SeMet was better absorbed than Na<sub>2</sub>SeO<sub>3</sub>. The protection of HaCaT from UVA-induced cell death was observed only with SeMet despite both forms increased glutathione

peroxidase-1 (GPX1) activities and selenoprotein-1 (SEPW1) transcript expression. After UVA irradiation, malondialdehyde (MDA) and SH groups were not modulated whatever Se chemical form. At toxic doses (100 μM SeMet and 5 μM Na<sub>2</sub>SeO<sub>3</sub>), Na<sub>2</sub>SeO<sub>3</sub> and SeMet inhibited cell proliferation associated with S-G2 blockage and DNA fragmentation leading to apoptosis caspase-3 dependant. SeMet only led to hydrogen peroxide production and to a decrease in mitochondrial transmembrane potential. Our study of the effects of selenium on HaCaT cells reaffirm the necessity to take into account the chemical form in experimental and intervention studies.

**Keywords** Selenomethionine · Sodium selenite · HaCaT cells · Antioxidant · UVA · Cell death

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## Abbreviations

DMSO	dimethylsulfoxide
CDK	cyclin dependant kinase
DCFDA	2',7'-dichlorofluorescein diacetate
DiOC6	3,3'-dihexyloxycarbocyanine iodide
FCS	foetal calf serum
GADD	growth arrest and DNA damage
GPX1	glutathione peroxidase 1
GSR	glutathione reductase
H <sub>2</sub> Se	selenide
HBSS	Hank's buffered salt solution
HPLC	high performance liquid chromatography
HPRT1	hypoxanthine phosphoribosyltransferase 1
ICP-MS	inductively coupled plasma mass-spectrometry
mTOR	mammalian target of rapamycin
MDA	malondialdehyde
MGG	May-Grünwald Giemsa
MI	mitotic index
MTP	mitochondrial transmembrane potential

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NHSF	normal human skin fibroblast
ROS	reactive oxygen species
SB	strand break
SeCys	selenocysteine
SeMet	selenomethionine
Na <sub>2</sub> SeO <sub>3</sub>	sodium selenite
SEPW1	selenoprotein W1
SH	thiol groups
SOD	superoxide dismutase
SP	selenoprotein
UVA	ultraviolet-A

## Introduction

Selenium is an essential trace element for humans, included in selenoproteins as the 21st amino acid Selenocystein (SeCys) [1]. Alternatively, SeMet can be incorporated non-specifically into proteins in place of methionine [2]. Thus, one of physiological functions of Se is thought to result from its existence in a number of selenoproteins in which Se is present as the amino acid selenocysteine. Se has been found (or predicted to be found) in 25 mammalian selenoproteins (for review see [3, 4]). SeCys is incorporated into the amino acid sequence of selenoprotein during translation, being coded for by a UGA codon in the coding region of mRNA [5]. The best characterized selenoproteins are the glutathione peroxidases (GPXs). Glutathione peroxidases (cytosolic GPX1, extracellular GPX3, phospholipid GPX4) have antioxidant functions through metabolism of hydrogen peroxide and some organic hydroperoxides in phospholipids (for review see [4]). Selenoprotein W1 (SEPW1) is ubiquitously expressed in tissues and its expression is regulated by selenium levels [6]. Moreover, SEPW1 has antioxidant properties [6, 7] and may be implied in cell cycle regulation [8–10]. The control of cell cycle progression plays a key role in terminal differentiation, growth and development. The connections between Se and the checkpoints at the G1/S and the G2/M transitions of the cell cycle have been reported [1, 9, 11–13].

Selenium has been shown in several studies to prevent the occurrence of some cancers [14]. The mechanisms that drive the selenium-anticancer action are not fully understood and may be led via antioxidant protection, enhanced immunity, regulation of cell proliferation, cell cycle modulation, apoptosis and inhibition of tumor cell invasion [1, 15].

Chemical form of selenium and selenium status at baseline may explain the discrepant results. The Nutritional Prevention of Cancer trial (NPC) [16] showed that Se, in the form of selenized yeast, in supra nutritional doses administered to

male humans, exhibited cancer chemoprevention effects against prostate cancer, whereas the SELECT trial [17], in which the Se was administered as SeMet, did not show the same beneficial effect. It has been suggested that two main factors may explain the difference. The first was the baseline Se levels of the studied population, the benefit being limited to people with the lowest baseline Se concentrations. The second was that Se-enriched yeast may contain active Se species whereas SeMet had limited therapeutic effect. This underlines the interest of understanding the biological activities and molecular targets of the Se chemical forms available in commercial Se supplements [18, 19].

Despite increasing incidence of skin malignancies, at least in part attributed to the deleterious effect of UVA-generated ROS, only few studies reported the association between Se and UVA related skin lesions.

Dreno et al. showed that Se did not prevent the occurrence of skin cancer linked to human papilloma virus in an organ transplant recipient population [20]. In mice, Pence et al. have reported a correlation between the selenium (as Na<sub>2</sub>SeO<sub>3</sub>) level in the diet and both the number of skin tumours and the activities of catalase, superoxide dismutase (SOD) and GPX1 [21]. Dietary SeMet protected against UV-light-induced skin damage in mice and reduced both inflammation and skin pigmentation [22].

In vitro, it has been shown that Se added into the cell culture medium protected skin cells from UVA- [23, 24] and UVB-induced [25] cytotoxicity in a dose and chemical form manner. The proposed mechanism was a modification of redox balance as well as selenoprotein synthesis and autophagy [24–27].

It was reported a generation of reactive oxygen species (ROS) by Na<sub>2</sub>SeO<sub>3</sub> [26, 28] as well as by SeMet [29] in cancer cells which triggered apoptosis. Apoptosis can occur by one or two representative pathways: the death receptor-mediated (extrinsic) pathway and the mitochondrial (intrinsic) pathway. Interaction of caspases plays an important role in either pathway. Caspase-3 belongs to executioner caspases (for review, see [30]). Apoptosis induction usually accompanies a loss of the mitochondrial transmembrane potential (MTP).

The aim of our experiments was to better understand the mechanisms involved in long term protective and deleterious effects of two selenium species (SeMet and Na<sub>2</sub>SeO<sub>3</sub>) found in commercially available supplements using HaCaT cell culture model submitted to UVA.

## Materials and Methods

### Chemicals

Seleno-L-methionine (SeMet, C<sub>5</sub>H<sub>11</sub>NO<sub>2</sub>Se) and sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>) were purchased from Sigma-Aldrich.

Stock solutions of SeMet and Na<sub>2</sub>SeO<sub>3</sub> were prepared in Milli-Q water, filter sterilized and stored at 4 °C.

### Cell Culture

HaCaT cells were propagated in RPMI-1640 supplemented with 10 % FCS (containing less than 20 nmol/l of Se), L-glutamine 2 mmol/l, penicillin 100 UI/ml and streptomycin 100 µg/ml (Life technologies, Saint Aubin, France), at 37 °C in a humidified atmosphere with 5 % CO<sub>2</sub>. Briefly, near confluent cells were trypsinized and plated at density of 1,000 cells/cm<sup>2</sup> in cultures dishes (BD Biosciences). Twenty four hours after plating, when cells were in exponentially growing cultures, the culture medium was replaced by fresh medium and the specific Se compound was added directly into the media giving specific final concentrations. Cells were next incubated for 144 h (6 days) to determine working doses. Then, using either non-toxic or toxic doses, cells were used to analyse several parameters described.

For MDA, SH groups, GPX and selenium absorption analysis, cells were washed three times in 0.2 M Tris, pH 7.3 (Sigma) and resuspended in 400 µl of Tris hypotonic (Tris 0.01 M), in which cells were submitted to 5 cycles of frost (liquid nitrogen)–defrost (37 °C) to obtain cell lysates. For RNA extraction, after the washing phases, cell dry pellets were frozen in liquid nitrogen and stored at –80 °C until analysis.

### Se Determination

Se was determined by ICP-MS (X serie II, Thermo Fisher Scientific, Waltham, MA) equipped with a Collision Cell interference elimination Technology (CCT) in cell lysates (see above) after dilution in nitric acid 1 % containing Gd as internal standard. The gas introduced in the CCT was a mixture of He and H<sub>2</sub>. The isotope ratio <sup>78</sup>Se/<sup>71</sup>Gd and an external calibration curve (16, 80 and 160 nmol Se/l) allowed the determination of selenium concentration expressed as nanomole per gram of total cell proteins.

### UVA Radiation

HaCaT cells were exposed to UVA irradiation, delivered from Tecimex apparatus (Dixwell, St Symphorien d'Ozon, France) with λ<sub>max</sub> at 372 nm and the relative spectral distribution of the lamp has been described in [31]. In order to establish which UVA dose significantly decrease the viability of HaCaT cells, dose kinetics were performed from 5 to 50 J/cm<sup>2</sup>. Dose to induce 30 % cell death was chosen: 23 J/cm<sup>2</sup>. For MDA and thiol group determinations, cells were harvested immediately after irradiation to avoid their repair. For GPX activity, cell cycle, apoptosis and MTT assays, cells were harvested 24 h after irradiation to allow

the cell to trigger a response. Cells were centrifuged at 300×g, 3 min.

### Cell Viability

Cell viability was determined colorimetrically using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, Saint Quentin Fallavier, France) assay [32]. Cell viability was reported as the percentage absorbance relative to the control. IC<sub>50</sub> (concentrations inducing 50 % cell death) values were determined by curve-fitting plots of cell viability versus Se compound concentration.

### Mitotic Index, Morphological Appearance and Microphotography

Optical micrographs of the cells in culture were obtained using the Leica inverted microscope (Wetzlar, Germany; ×400) and cells were trypsinized and washed twice in Hank's Buffered Salt Solution (HBBS). Mitotic index (MI) was determined by using cells fixed on glass slides by cytospin apparatus (Shandon, Pittsburgh, PA) with further May–Grünwald Giemsa (MGG) staining. The MI was determined as the percentage of mitotic figures of 300 cells from each sample with conventional analysis using a Zeiss microscope (Oberkochen, Germany) as previously described [33]. Microphotographs were taken using a Canon Power Shot S50 digital camera (Canon, Courbevoie, France).

### Cell Cycle, Apoptosis and Caspase-3 Activity Determinations

Cell DNA content was analysed with IP staining using the CycleTest™ PLUS/DNA reagent kit (Biosciences, San Jose, CA) after 6 days selenium supplementation, according to manufacturer recommendations. Data were collected on FACS Canto II flow cytometer (Becton Dickinson, San José, CA) using FACS DIVA software (Biosciences) and the phases of the cell cycle were determined. Apoptosis was evaluated as the percentage of cells in the sub-G<sub>0</sub>/G<sub>1</sub> phase then confirmed using the Annexin V-FITC/PI method (Vybrant™ Apoptosis Assay kit, Molecular Probes, Eugene, OR) using flow cytometry analysis. The role of caspase-3 in the apoptotic process was determined by the Caspase-3 Fluorometric Assay (R&D Systems, Minneapolis, MN) as described by the manufacturer.

### Measurement of Transmembrane Mitochondrial Potential (MTP)

Na<sub>2</sub>SeO<sub>3</sub> or SeMet HaCaT treated cells were stained with 40 nM of 3,3'-dihexyloxycarbocyanine iodide (DiOC6)

(Molecular Probes, Eugene, OR) at 37 °C for 30 min. Cells were washed twice with PBS and analysed by flow cytometer [34]. The MTP was expressed as the percentage of cells showing a decrease in the fluorescence intensity as determined using FACS Diva software (Biosciences).

#### Measurement of Intracellular Hydrogen Peroxide

Intracellular hydrogen peroxide was detected by means of an oxidation-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR). DCFDA was deacetylated by nonspecific esterase (NSE) then oxidized by a peroxide to a fluorescent compound (DCF). Briefly, trypsinized cells were incubated with DCFDA (at a final concentration of 5 µM) at 37 °C for 30 min then washed twice with PBS and analysed by flow cytometer. Hydrogen peroxide production was expressed as the percentage of cells showing an increase in the fluorescence intensity as determined using FACS Diva software (Biosciences).

#### GPX1 Activity

GPX1 activity was measured using the glutathione reductase (GSR)-NADPH method [35]. GPX1 activity was expressed in terms of nanomoles of NADPH oxidized per minute per milligram of protein.

#### SEPW1 RT-q-PCR

Total RNA extraction was performed with RNeasy Mini Kit (Qiagen, Courtaboeuf, France) following manufacturer recommendations. Total RNA was treated with RNase-free DNase I (Qiagen, Courtaboeuf, France). cDNA was reverse transcribed from 1 µg of total RNA with the SuperScriptIII First-Strand Synthesis (Life Technologies, Saint Aubin, France). As recommended, an RNase H treatment was added.

Real time RT-PCR was conducted using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Stratagene (Mx3005P, La Jolla, CA). The primers (5'-3') SEPW1 sense GCCGTCCGAGTCGTTTATTGT antisens GGCTACCAT CACTTCAAAGAACC (Tm 60 °C, 400 nM) and HPRT1 sense CTCATGGACTGATTATGGACAGGAC antisense GCAGGTCAGCAAAGAACTTATAGCC (Tm 60 °C, 400 nM) were chosen to include intron spanning. Primers were synthesized by Life Technologies.

Gene expression was quantified using the comparative threshold cycle (Ct) method [36]. The amount of target gene, normalized to an endogenous reference gene (HPRT1), was expressed relative to the control cells (without Se supplementation).

#### Malondialdehyde Measurement

MDA, an end product of lipid peroxidation process [37], was determined using the separation of MDA-thiobarbituric acid (TBA) adducts on reversed-phase HPLC as previously described [38]. The results were expressed as micromole per gram of total cell proteins.

#### Quantitative Determination of Thiol Groups

Protein oxidation protection was evaluated by thiol groups (SH) determination [39, 40]. The results were expressed as micromole per gram of total cell proteins.

#### Quantitative Protein Determination

Protein levels in total cell lysates and in soluble fractions were determined using the BCATM Bincinchoninic acid kit (Interchim, Montluçon), according to manufacturer recommendations.

#### Statistical Analysis

Results are expressed as means±SD for the number of experiments indicated. All statistical analysis of data was computed using StatView (SAS Institute, USA). The sources of variation for multiple comparisons were assessed by ANOVA. The differences were considered statistically significant at  $p < 0.05$ .

## Results

### Absorption of Selenium Depended on the Chemical Form

In order to evaluate the capacity of cells to absorb Se as a function of its chemical form, Se content was determined in cell lysates (Table 1). A negative relationship was observed between absorption and selenium concentration supplementations (for SeMet,  $p = 0.0017$  and  $p = 0.0001$  for Na<sub>2</sub>SeO<sub>3</sub> in the lysates). In addition, Se from Na<sub>2</sub>SeO<sub>3</sub> was poorly absorbed compared to SeMet.

### Selenium Toxicity Differed as a Function of Dose and Chemical Form

SeMet was better tolerated than Na<sub>2</sub>SeO<sub>3</sub> in HaCaT cells (Fig. 1a). Indeed, the IC<sub>50</sub> of SeMet is about 24-fold higher than IC<sub>50</sub> of Na<sub>2</sub>SeO<sub>3</sub> (55.4 µM versus 2.3 µM). Two working zones were determined to better understand biological effects of Se: a non-toxic and a toxic zone. The working doses were 1 µM Na<sub>2</sub>SeO<sub>3</sub> and 10 µM SeMet for the non-

**Table 1** Selenium absorption: The percentage of selenium absorption was calculated as a function of selenium found in HaCaT cell lysates after 144 h incubation with variable doses of SeMet or Na<sub>2</sub>SeO<sub>3</sub>

Absorption of selenium added (%)		
μM <sup>a</sup>	SeMet	Na <sub>2</sub> SeO <sub>3</sub>
0.1	22.07±2.31	3.22±1.23
0.5	20.16±2.02	0.73±0.29
1	17.23±2.09	0.47±0.19
5	15.19±1.87	nd-toxic
10	14.48±2.93	nd-toxic

Results are mean±SD of  $n > 4$  independent experiments

<sup>a</sup> Concentrations added in cell culture medium

toxic dose and 5 μM Na<sub>2</sub>SeO<sub>3</sub> and 100 μM SeMet which represented 80 % toxicity.

The toxicity of selenium compounds can be visualized by cell morphology changes, as seen by photographs of cytocentrifuged HaCaT after MGG staining (Fig. 1b). HaCaT cells at not toxic doses (c and e, respectively) were morphologically comparable to the control (a). On the contrary, at toxic doses (5 μM Na<sub>2</sub>SeO<sub>3</sub> and 100 μM SeMet), HaCaT cells exhibited a cytoplasmic vacuolization and detachments from dishes (i and g). Nevertheless, SeMet morphological changes were related to autophagy (data not shown) whereas Na<sub>2</sub>SeO<sub>3</sub> treatment induced apoptosis. MGG staining showed that few cells Na<sub>2</sub>SeO<sub>3</sub>- and SeMet-treated showed mitotic figures (MI=0.2 in control vs 0.6 and 1.2 for 5 μM Na<sub>2</sub>SeO<sub>3</sub> and 100 μM SeMet, respectively) (b, j and h).

DNA fragmentation was induced after 144 h treatment in HaCaT cells by SeMet at 100 μM (20 % in average) and Na<sub>2</sub>SeO<sub>3</sub> at 5 μM (25 % in average; Fig. 1c).

#### Cell Death Process Characterization: Hydrogen Peroxide, MTP and Caspase-3 Analysis

In order to characterize Se toxicity causing, ROS production, MTP and cell death, flow cytometry analyses after propidium iodide, DCFDA, DiOC<sub>6</sub> or Annexin V-FITC/PI staining were respectively performed. Moreover, to better describe apoptosis Se-induced, caspase-3 activity was determined after 144 h treatment.

After a 144-h treatment in HaCaT cells, DNA fragmentation was associated with hydrogen peroxide production (Fig. 2a) and a decrease in MTP (Fig. 2b) when using SeMet at 100 μM but not Na<sub>2</sub>SeO<sub>3</sub> at 5 μM. Both forms induced apoptosis (Fig. 2c) and caspase-3 activity (Fig. 2d). With SeMet, necrosis was also involved although to a lesser extent.

#### SeMet Protected HaCaT Cells from UVA-Induced Toxicity

The UVA dose was chosen to induce 20.6±3.3 % cell toxicity 24 h after UVA exposure, i.e. 23 J/cm<sup>2</sup> in HaCaT cells (Fig. 3). At non-toxic doses, only HaCaT supplementation with SeMet protected HaCaT from the UVA-radiation inducing cell death in a dose independent way (Fig. 4a). Na<sub>2</sub>SeO<sub>3</sub> supplementation did not exhibit protection toward mortality-UVA induced (Fig. 4a).

#### SeMet and Na<sub>2</sub>SeO<sub>3</sub>-Induced GPX Activity

At non-toxic doses, GPX activity was induced by both SeMet and Na<sub>2</sub>SeO<sub>3</sub> treatment. Using toxic doses, GPX activity was not detected (Fig. 4b) due to a cell death process.

#### SeMet and Na<sub>2</sub>SeO<sub>3</sub>-Induced SEPW1 Transcript Expression

At non-toxic doses, SEPW1 transcript expression was significantly induced by both components (Fig. 4c). At toxic dose, SEPW1 transcript was not detected.

#### SeMet and Na<sub>2</sub>SeO<sub>3</sub> Did Not Modulate MDA Levels at Baseline Nor SH Group Levels

At baseline (without UVA), MDA levels were not changed by the supplementation with either non-toxic doses of SeMet (0.15±0.01 μmol/mg proteins in control cells versus 0.14±0.01 μmol/mg proteins in cells supplemented with 10 μM SeMet) or Na<sub>2</sub>SeO<sub>3</sub> (0.15±0.04 μmol/mg proteins in control cells versus 0.13±0.04 μmol/mg proteins in cells supplemented with 1 μM Na<sub>2</sub>SeO<sub>3</sub>). When cells were UVA-irradiated, MDA significantly increased but neither the Na<sub>2</sub>SeO<sub>3</sub> nor SeMet supplementation had any effect to protect cells from UVA-deleterious effect on lipid damages (irradiated untreated cells, 0.18±0.04 μmol/mg proteins; irradiated cells treated by 1 μM Na<sub>2</sub>SeO<sub>3</sub>, 0.14±0.02; irradiated cells treated by 10 μM SeMet, 0.13±0.03 μmol/mg proteins).

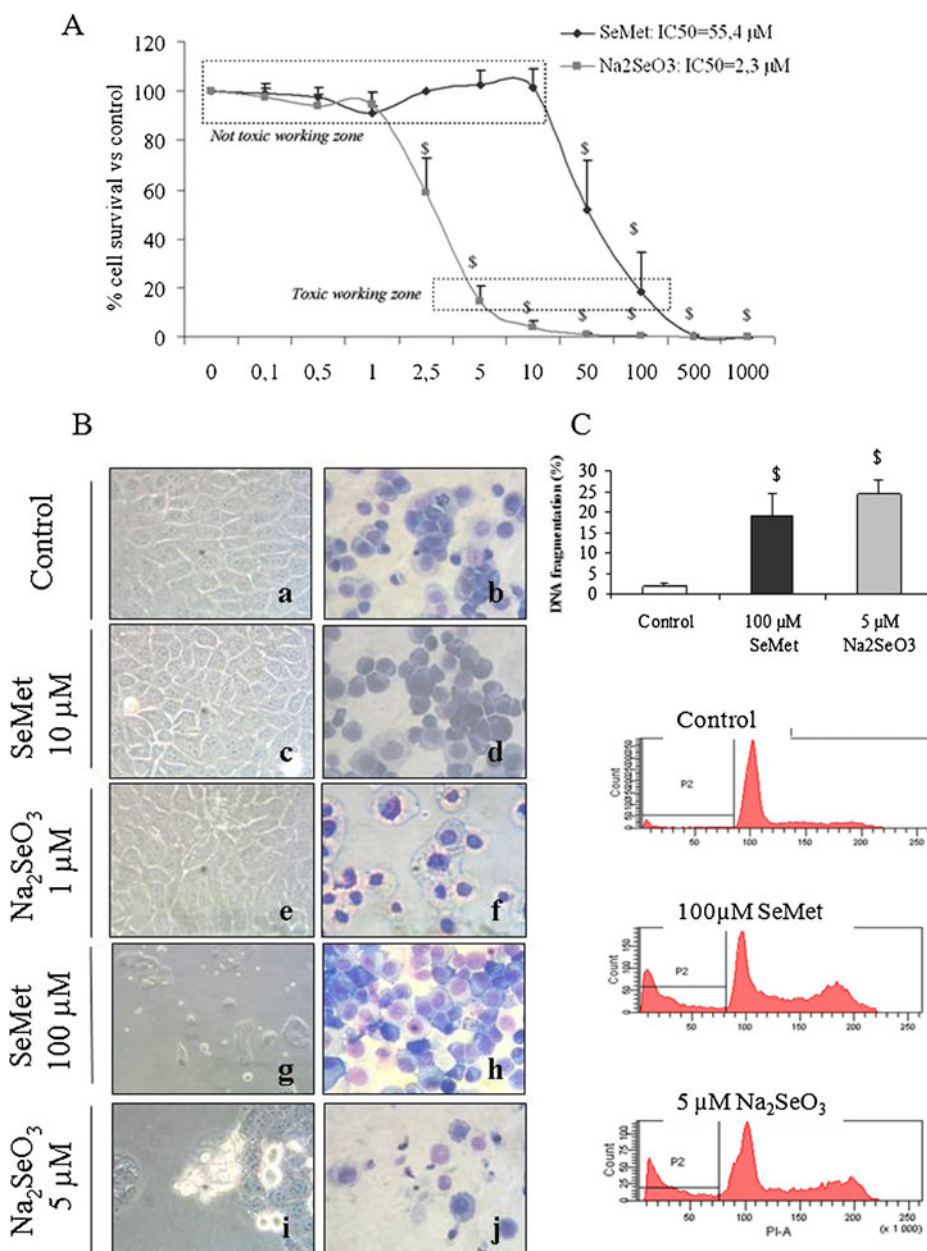
SH-group concentrations did not vary after SeMet or Na<sub>2</sub>SeO<sub>3</sub> supplementation at baseline (137.8±20.0 μmol/mg proteins in controls cells versus 125.3±16.5 μmol/mg proteins in cells treated by 10 μM SeMet and 135.7±6.9 μmol/mg proteins in cells treated by 1 μM Na<sub>2</sub>SeO<sub>3</sub>). After UVA irradiation, the percentage of SH groups remaining was similar in supplemented HaCaT cells whatever Se chemical form (141.0±22.8 μmol/mg proteins in cells irradiated and not treated).

#### Cell Cycle Modulation

Cell cycle analysis was performed following propidium iodide staining using flow cytometry (Table 2). As showed above, MGG staining illustrated that very few cells SeMet

**Fig. 1** Toxicity of 144 h selenium supplementation as a function of dose and selenium form ( $\text{Na}_2\text{SeO}_3$  and SeMet) in HaCaT cell. **a** Survival (%) was measured using the MTT assay to calculate the IC50 values.

Two working doses to study antagonist Se biological effects were determined: a non-toxic zone (end of the plateau) and a toxic zone representing about 80 % cell death. **b** Optical microphotographs ( $\times 400$ ) illustrated morphology of the HaCaT cells either in culture (*a*, *c*, *e*, *g* and *i*) or after MGG staining of cytoentrifuged cells (*b*, *d*, *f*, *h* and *j*). HaCaT cells were cultured without treatment (*a/b*) or treated with either SeMet or  $\text{Na}_2\text{SeO}_3$  at non-toxic dose (10 and 1  $\mu\text{M}$ , *c/d* and *e/f*, respectively) or at toxic dose (100 and 5  $\mu\text{M}$ , *g/h* and *i/j*, respectively). Note the cell detachments and hypervacuolization in the cytoplasm after 5  $\mu\text{M}$   $\text{Na}_2\text{SeO}_3$ . Cell density decrease and floating cells were observed for 100  $\mu\text{M}$  SeMet. Cells in *c* and *e* were not significantly different from the control. **c** DNA fragmentation was determined by flow cytometry (IP staining). P2 represents cells in the sub-G0/G1 phase (DNA fragmentation). Results are mean  $\pm$  SD of  $n > 3$  independent experiments with  $^{\$}p < 0.0001$  versus the control (not supplemented cells)



and  $\text{Na}_2\text{SeO}_3$  treated showed mitotic figures suggesting that the blockage was in the G2 phase of the cell cycle (Fig. 1b (h, j), respectively).

HaCaT cells treated with 10 and 100  $\mu\text{M}$  SeMet were arrested in the S/G2 phase of the cell cycle. At non-toxic dose (10  $\mu\text{M}$ ), this arrest was not associated neither with apoptosis nor necrosis (1.2 %  $\pm$  0.9 necrotic and 8.4 %  $\pm$  3.2 apoptotic cells not treated versus 0.6 %  $\pm$  0.3 necrotic and 5.2 %  $\pm$  1.8 apoptotic SeMet treated cells) whereas it was so at the toxic dose (100  $\mu\text{M}$  SeMet) as shown in Fig. 2c.

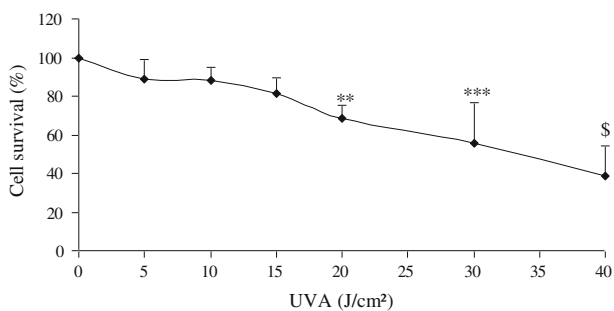
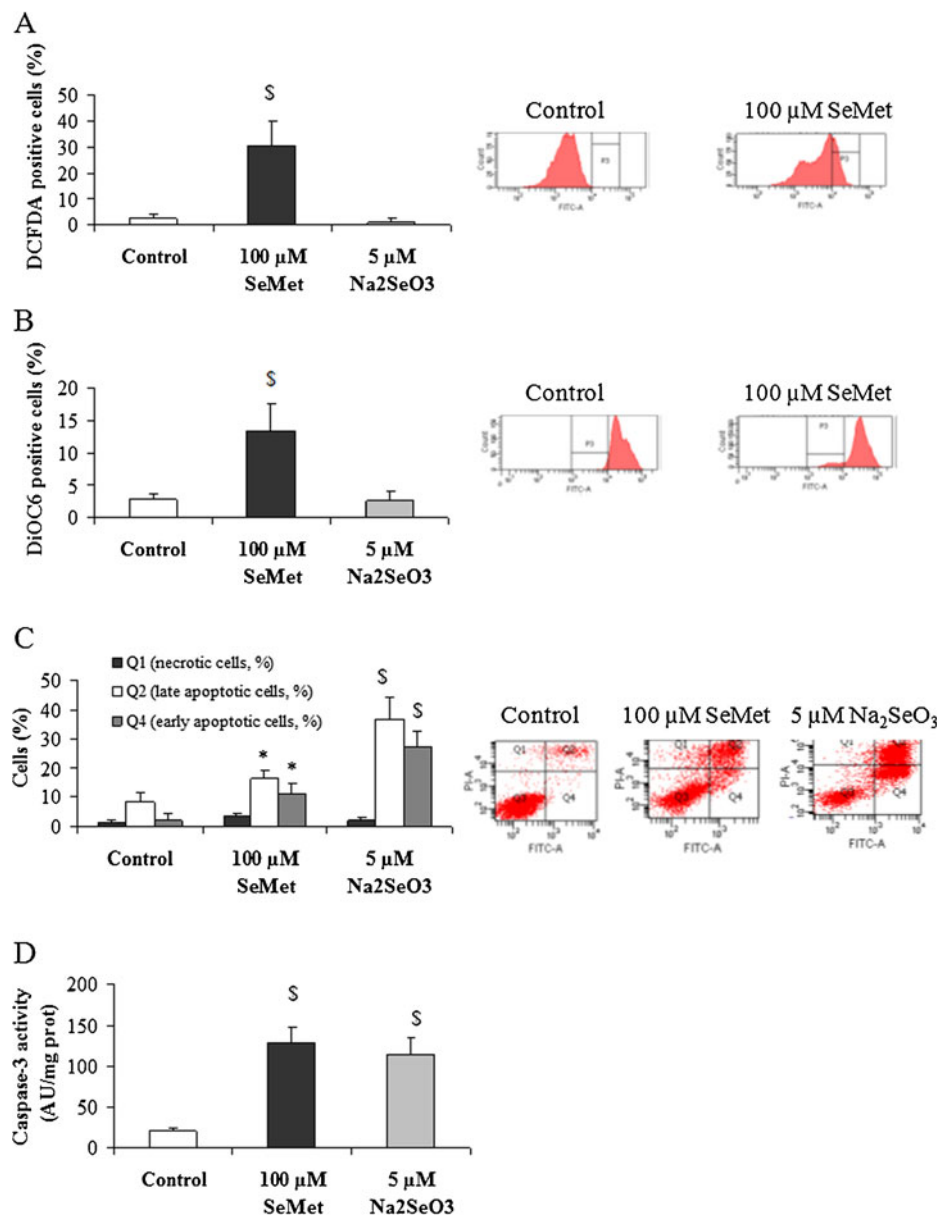
$\text{Na}_2\text{SeO}_3$  at 5  $\mu\text{M}$  arrested HaCaT cells in the S/G2 phase whereas no effect was observed at non-toxic dose (1  $\mu\text{M}$ ). The arrest observed at 5  $\mu\text{M}$   $\text{Na}_2\text{SeO}_3$  was associated with apoptosis and necrosis as shown in Fig. 2c.

## Discussion

The data presented herein support the hypothesis that  $\text{Na}_2\text{SeO}_3$  (inorganic) and SeMet (organic) compounds have different metabolisms and distinct modes of actions in inducing apoptosis, modulating the cell cycle and triggering antioxidant responses.

In this study, HaCaT immortalized keratinocytes cells were chosen as they are a well-accepted paradigm for an early stage of skin cancer development [41]. This model is thus relevant to study antioxidant Se properties in parallel to its toxic activities. Previous studies have reported the antioxidant properties of both SeMet and  $\text{Na}_2\text{SeO}_3$  as well as  $\text{Na}_2\text{SeO}_3$  toxicity using different cell models but to our knowledge this is the first report dealing with SeMet toxicity. In addition, in contrast to many

**Fig. 2** Se cell death process characterization. HaCaT cells were cultured for 144 h without treatment or treated with either  $\text{Na}_2\text{SeO}_3$  at 5  $\mu\text{M}$  or SeMet at 100  $\mu\text{M}$ . HaCaT cells were then trypsinized and used for **a** hydrogen peroxide production (DCFDA staining), **b** membrane mitochondrial potential ( $\text{DiOC}_6$  staining), **c** cell death (annexin V-FITC/PI) using flow cytometry analysis and **d** caspase-3 activity using fluorometric method. For hydrogen peroxide production and MTP studies, *P3* represents positive cells. For Annexin V-FITC/PI analysis: Q3 double-negative annexin V and PI represents viable cells, Q1: PI-positive represents necrotic cells, Q2: double-positive cells represent late apoptotic and necrotic cells, Q4: annexin V-positive and PI negative represents early apoptotic cells. Results are means of  $n \geq 3$  independent experiments  $\pm$ SD with  $*p < 0.05$  and  $^{\$}p < 0.0001$  vs control

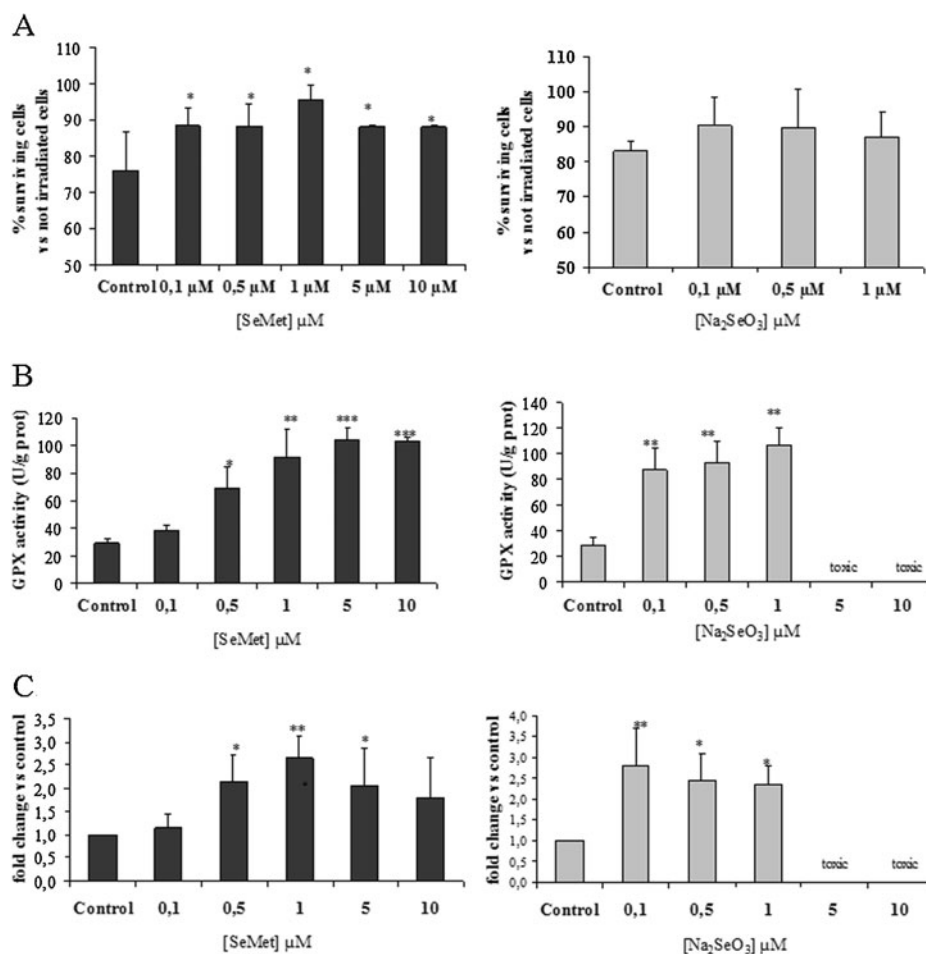


**Fig. 3** UVA irradiation induced-HaCaT cells toxicity was determined using a MTT assay. Results are mean  $\pm$ SD of  $n > 3$  independent experiments with  $**p < 0.00$ ,  $***p < 0.0005$  and  $^{\$}p < 0.0001$  versus the control (not irradiated cells)

published studies, our aim was to mimic long term supplementation using a 6-day-supplementation period. Finally, HaCaT cells have never been evaluated.

It is noted that Se uptake from  $\text{Na}_2\text{SeO}_3$  was very low in HaCaT cells which was comparable to that observed with PC-3 and Jurkat cells [42]. The low Se uptake could be due to an adaptative response of the cells to the higher toxicity of  $\text{Na}_2\text{SeO}_3$  compared to that of SeMet. Indeed, several studies on selenium absorption across intestinal barrier models have shown a net absorptive flux across the Caco-2 cell monolayer. This suggested that a transporter-mediated transcellular pathway was involved for SeMet, while a paracellular pathway for  $\text{Na}_2\text{SeO}_3$  was preferred, which is supported by the low intracellular concentration [13, 43, 44]. Moreover, the metabolism of Se also depends on which form it is ingested [45]. In

**Fig. 4** Protective effects of 144 h selenium supplementation on HaCaT cells were evaluated by the analysis of **a.** cell viability after UVA irradiation. HaCaT cells were cultured without treatment or Se supplemented at non-toxic doses, then exposed to UVA radiation. Viability was determined 24 h after 23 J/cm<sup>2</sup> UVA-inducing about 21 % cell death. The control cells were not supplemented and irradiated (**b**). GPX activities (**c**). SEPW1 transcript expression analysis by RT-q-PCR, normalized with HPRT1. Results are means of  $n > 3$  independent experiments  $\pm$ SD with \* $p < 0.05$ , \*\* $p < 0.005$  and \*\*\* $p < 0.0005$  versus the control (cells not supplemented)



humans, the organic food forms of Se are better absorbed than the inorganic forms [46, 47] even though they are transformed into the selenide state [1] prior to their incorporation into the proteins [5]. However, our results demonstrated that the small

**Table 2** Cell cycle analysis

	G0/G1	S	G2/M
SeMet ( $\mu$ M)			
0	70.4 $\pm$ 2.8	15.2 $\pm$ 1.8	13.1 $\pm$ 1.4
10	63.15* $\pm$ 3.6	21.1* $\pm$ 2.9	13.9 $\pm$ 0.8
100	47.7** $\pm$ 4.2	26.0*** $\pm$ 2.9	26.2*** $\pm$ 4.2
Na <sub>2</sub> SeO <sub>3</sub> ( $\mu$ M)			
0	68.0 $\pm$ 3.1	14.1 $\pm$ 2.5	15.7 $\pm$ 5.5
1	65.8 $\pm$ 1.6	14.3 $\pm$ 3.9	16.7 $\pm$ 3.3
5	40.4*** $\pm$ 0.8	26.4*** $\pm$ 3.8	31.7*** $\pm$ 3.0

The cell cycle distribution was expressed in percentage of HaCaT cells in each phase of the cell cycle 144 h after exposure to not toxic doses (10  $\mu$ M SeMet and 1  $\mu$ M Na<sub>2</sub>SeO<sub>3</sub>) and to toxic doses (100  $\mu$ M SeMet and 5  $\mu$ M Na<sub>2</sub>SeO<sub>3</sub>). To harmonize the results, sub-G0/G1 phase has been eliminated. Results expressed are the mean of  $n \geq 3$  independent experiments  $\pm$ SD with \* $p < 0.05$ , \*\* $p < 0.0001$  and \*\*\* $p < 0.0005$  versus the control (not supplemented cells)

amount of Na<sub>2</sub>SeO<sub>3</sub> entering into the cells was sufficient to induce important increases of GPX1 and SEPW1 and cell death by apoptosis, via caspase-3, depending on the concentration. Although Na<sub>2</sub>SeO<sub>3</sub> is rarely present in foods [48], it is also reduced metabolically to selenide and converted enzymatically to SeCys. Accordingly, these inorganic Se species can act like SeCys to supply Se for SP synthesis. Apart from their different mechanisms of absorption, the metabolic fates of these forms of Se are quite similar, due to their conversion to selenide and SeCys prior to their incorporation into SP [1].

At sub-toxic doses, both Na<sub>2</sub>SeO<sub>3</sub> and SeMet exhibit antioxidant properties, and both SeMet and Na<sub>2</sub>SeO<sub>3</sub> optimized the SEPW1 transcript expression and GPX1 biosynthesis, but only SeMet protected HaCaT from UVA-dose-induced cytotoxicity. Rafferty et al. showed that SeMet conferred substantial protection from high UVB-dose-induced cytotoxicity in keratinocytes [25]. The same authors showed that the protective effect was more modest and required higher Se dosages when the supplementation occurred prior to, than after UV irradiation. Other authors reported that Na<sub>2</sub>SeO<sub>3</sub> increased the survival of UVA-irradiated fibroblasts [23].

Interestingly, neither SeMet nor Na<sub>2</sub>SeO<sub>3</sub> significantly affected the MDA concentration. Na<sub>2</sub>SeO<sub>3</sub> at 0.58  $\mu$ M has been



shown to reduce the levels of thiobarbituric acid-reacting substances (TBARS), another lipid peroxidation marker, in fibroblasts [23]. Moreover, these data suggest that the UVA-protection can be due to the Se incorporation into SP causing an enhancement of the GPX1 activity and the levels of SEPW1 transcript expression rather than a direct antioxidant effect, which could explain why the levels of MDA were not decreased by both Se treatments chosen. It is worth to be noted that the concentration of the protein SH-groups remained unchanged by addition of non-toxic doses of both SeMet and Na<sub>2</sub>SeO<sub>3</sub> or by UVA irradiation despite it has been demonstrated that selenite reacts immediately and directly with thiols, simultaneously generating superoxide [49, 50].

Although both compounds conferred antioxidant protection, the window range of protection was higher for SeMet.

Selenium is essential for the growth of cells in culture [51] but with a relatively narrow window of tolerance [12]. In the same time, Se inducing cell cycle arrest can be a mechanism arising before apoptosis caused by non-specific genotoxic effects manifested by single strand breaks and cytotoxicity [52–54], as shown in this study. At non-toxic dose, SeMet induced an arrest in S/G2 which was not observed for Na<sub>2</sub>SeO<sub>3</sub>, suggesting another cell cycle regulatory process. Previous studies have reported a significant effect of the chemical form on the metabolic pathways [52–55]. At toxic dose, Na<sub>2</sub>SeO<sub>3</sub> and SeMet led to an arrest in the S-G2/M phases. This blockage was rather in the G2 phase, (as confirmed by the MGG staining) possibly in response to ROS-inducing DNA fragmentation and then apoptosis, as shown in previous studies [11, 53]. This arrest has been associated with increased expression of Growth Arrest DNA damage (GADD) -45 and -34 [11], a decrease in cyclins D1, E and A which bound cycline-dependant-kinases (cdk) [53]. Some studies showed that organic Se compounds (i.e. methylselenocysteine) and their metabolites (i.e. methylselenol) rather induced a G1 cell cycle arrest associated with a modulation of cell cycle actors [53, 56, 57], such as CDKN1C/p57<sup>KIP2</sup> [58] suggesting that methylselenol have potent anti-cancer properties [57, 59, 60]. Despite degradation of SeMet to methylselenol by  $\gamma$  lyase action has been reported [61], we did not evidence any arrest in G1 under SeMet supplementation.

Another actor in cell cycle modulation by selenium may be SEPW1. We showed that SEPW1 transcript expression was increased in both Na<sub>2</sub>SeO<sub>3</sub>- and SeMet-treated cells (at non-toxic doses). In this work, we did not focus on a correlation between cell cycle arrest and SEPW1 expression but some studies showed that its depletion induced a G1 arrest in non-tumorigenic and cancer cells lines [8–10] that may be mediated by p53 and p21 [10].

It is well known that organoselenium compounds are less toxic than inorganic compounds. Indeed, in this study, Na<sub>2</sub>SeO<sub>3</sub> caused apoptosis in HaCaT cells when present at a concentration approximately 24 times lower than SeMet.

Our results suggest that the reported anti-tumor effects of Na<sub>2</sub>SeO<sub>3</sub> [26, 28, 30, 62–64] and SeMet may be due to its toxicity. Moreover, we established that HaCaT cells were able to absorb more Se than normal human skin fibroblast (0.5 % for Na<sub>2</sub>SeO<sub>3</sub> and 3.5 % for SeMet, unpublished data) showing that immortalized cells may be more sensitive to Se toxicity than normal cells.

We illustrated that Na<sub>2</sub>SeO<sub>3</sub> induced the cell death process in parallel to DNA fragmentation, cell cycle arrest (in S/G2) and apoptosis (via caspase-3). Previous reports showed that the subsequent apoptosis to Na<sub>2</sub>SeO<sub>3</sub>-induced-DNA damage followed different mechanisms of action [52–54, 65, 66]. Among them, ROS mediated the activation of caspases [26, 67]. This may directly imply the reduced form of selenite, the selenide, which, in sufficient concentrations, with oxygen and thioredoxin reductase is able to form ROS. This reaction is considered to be a mechanism of Se toxicity in which Se is transformed from antioxidants to prooxidants [68]. Nevertheless, hydrogen peroxide generation and MTP decrease were not detected at 30 min and 1, 2, 4, 24, 48 and 144 h Na<sub>2</sub>SeO<sub>3</sub> treatment (data not shown). This suggests that in HaCaT cells, hydrogen peroxide may not be the main ROS involved in Na<sub>2</sub>SeO<sub>3</sub>-induced apoptosis. Indeed, previous report have documented that Na<sub>2</sub>SeO<sub>3</sub> generated hydrogen peroxide ROS [26, 28] (peak at 2 h treatment for 20 and 6  $\mu$ M respectively) but also anion superoxide [62] (peak at 6 h for 7  $\mu$ M treatment) in different cancer cells. Moreover, it is reported that selenium compounds induced mitochondrial permeability transition [55, 62].

Even though the exact mechanism of ROS generated by SeMet is unknown, we showed here that SeMet was able to generate hydrogen peroxide, DNA fragmentation and apoptosis via caspase-3, in the same proportion than Na<sub>2</sub>SeO<sub>3</sub>. It was found that mTOR activation signaling pathway and Akt played a pro-apoptotic role in SeMet induced-apoptosis [29]. Currently, we are investigating the mechanisms triggering apoptosis process, identifying apoptosis actors in HaCaT cells and the role of autophagy induced by Na<sub>2</sub>SeO<sub>3</sub> and SeMet.

In summary, in a review of the mechanisms for selenium's cancer preventive activity [59], it has been stated in 1999: "although it is convenient to describe the effects of selenium in terms of the element, it must always be kept in mind that the chemical form and dose are determinants of its biological activities as an essential nutrient, cancer preventive agent, or toxicant". Our results corroborate this statement, emphasize the importance of considering not only the dose but also the chemical form in the interpretation of results and provided new insights into the mechanism of SeMet toxicity and the importance of ROS, MTP and caspase-3 in driving apoptotic death in HaCaT cells. However it is difficult to generalize our results to other cell types or to other operating conditions. SeMet should be tested in skin cancer cell lines to observe its potential anti-cancer effects.

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