Se-methylselenocysteine protects human hepatoma HepG2 cells against oxidative stress induced by tertbutyl hydroperoxide.

Susana Cuello[1], Sonia Ramos[2], Raquel Mateos[2], M. Angeles Martín[2], Yolanda Madrid[1], Carmen Cámara[1], Laura Bravo[2] and Luis Goya[2]*

[1]Departamento de Química Analítica, Facultad de Químicas, Universidad Complutense, 28040-Madrid.

[2]Departamento de Metabolismo y Nutrición; Instituto del Frío (CSIC); José Antonio Novais, 10; 28040 Madrid, Spain

Running title: Se-methylselenocysteine protects HepG2 cells from oxidative stress.

*Corresponding author: Luis Goya, Departamento de Metabolismo y Nutrición, Instituto del Frío (CSIC); José Antonio Novais, 10; 28040 Madrid, Spain

Phone: +34.91.544.56.07
Fax: +34.91.549.36.27

e-mail: luisgoya@if.csic.es

1   **ABSTRACT**

2   Se-methylselenocysteine (Se-MeSeCys) is a common selenocompound in the

3   diet with a tested chemopreventive effect. This study investigated the potential

4   protective effect of Se-MeSeCys against a chemical oxidative stress induced by tert-

5   butylhydroperoxide (t-BOOH) on human hepatoma HepG2 cells. Speciation of

6   selenium derivatives by LC-ICP-MS depicts Se-MeSeCys as the only selenocompound

7   in the cell culture. Cell viability (lactate dehydrogenase) and markers of oxidative

8   status: concentration of reduced glutathione (GSH) and malondialdehyde (MDA),

9   generation of reactive oxygen species (ROS) and activity of antioxidant enzymes

10 glutathione peroxidase (GPx) and glutathione reductase (GR) were evaluated. Pre-

11 treatment of cells with Se-MeSeCys for 20 h completely prevented the enhanced cell

12 damage, MDA concentration and GR and GPx activity and the decreased GSH induced

13 by t-BOOH but did not prevent increased ROS generation. The results show that

14 treatment of HepG2 cells with concentrations of Se-MeSeCys in the nanomolar-

15 micromolar range confers a significant protection against an oxidative insult.

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18 <u>Keywords</u>: antioxidant defences, biomarkers for oxidative stress, dietary antioxidants,

19 selenium compounds

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INTRODUCTION

Human exposure to potentially toxic chemicals, either through an occupational environment, or as the result of plant and foodstuff pyrolysis (e.g. tobacco smoke, charbroiled foods) is almost unavoidable. In many instances, increased exposure to these hazardous chemicals, many of which are prooxidants or procarcinogens, is linked to an increased incidence of cardiovascular disease and cancer [1,2]. This has prompted a search for diets or chemical supplements that might mitigate or prevent the toxic outcome of exposure. There is substantial evidence that antioxidative food components have a protective role against oxidative stress-induced atherosclerosis, degenerative and age-related diseases, cancer and aging [3,4]. Among dietary compounds considered for chemopreventive activity, selenium showed early and continued promise [1,2,4-8]. Selenium is a trace element essential to human health found in fish, meat and plants such as garlic, onion and broccoli, and a deficiency of this element induces pathological conditions, such as cancer, coronary heart disease, and liver necrosis [6,9]. Garlic is the most popular and well-researched *Allium* plant that is known to accumulate Se as selenoamino acid derivatives, including Se-methyl-l-selenocysteine (Se-MeSeCys), one of the major forms of selenium in the diet, and glutamylmethylselenocysteine (GluMeSeCys) [9,10].

Selenium compounds have been widely reported to be effective chemopreventive agents against multiple models of tumorigenesis [4-8,11-14]. These protective effects of selenium seem to be primarily associated with its presence as a cofactor in the enzymes glutathione peroxidase (GPx) and thioredoxin reductase, which are known to protect cellular components from oxidative damage [1,2]. Although these properties indicate that Se-MeSeCys may favourably affect the antioxidant defence

3

45 system [1,2], little is known about the potentially beneficial role of Se-MeSeCys against

46 oxidative damage in vivo, both in cultured cells and live animals.

47   Human hepatocarcinoma HepG2 is widely used for biochemical and nutritional

48 studies as a cell culture model of human hepatocytes since they retain their morphology

49 and most of their function in culture [15]. In addition, HepG2 is a reliable model where

50 many dietary antioxidants and conditions can be assayed with minor interassay

51 variations [16-20]. Previous studies from our laboratory have shown that the plant

52 flavonol quercetin [18], the olive oil phenol hydroxytyrosol [19], and or a digested

53 coffee melanoidin [20] elicit a favorable response of the antioxidant defense system in

54 cultured human hepatoma HepG2 cells. Therefore, the potential protective effect of

55 different concentrations of the dietary compound Se-MeSeCys against an oxidative

56 stress chemically induced by a potent prooxidant, tert-butyl hydroperoxide (t-BOOH),

57 has now been tested in cultures of HepG2. Cell integrity and several markers of

58 oxidative status such as concentration of reduced glutathione (GSH), generation of

59 reactive oxygen species (ROS), evaluation of the activity of antioxidant enzymes GPx

60 and glutathione reductase (GR) and determination of malondialdehyde (MDA) as a

61 biomarker of lipid peroxidation, were measured to estimate the effect of Se-MeSeCys in

62 cell survival and the response of the antioxidant system of HepG2 to t-BOOH.

63

64 **MATERIALS AND METHODS**

65 **Reagents and samples**

66   Se-MeSeCys (Sigma Chemicals, St. Louis, MO, USA) was dissolved in Milli-Q

67 water. Inorganic selenium solution was obtained by dissolving sodium selenite (Merck,

68 Darmstadt, Germany) in deionised Milli-Q water. Stock solutions of 10 mg/L were

69 stored in the dark at 4 ^{o}C and working standard solutions were prepared daily by

dilution. For hydride generation atomic fluorescence spectroscopy (HG-AFS) studies, sodium borohydride (Sigma-Aldrich, Steinheim, Germany) was prepared in 0.3 % sodium hydroxide. For high-performance liquid chromatography (LC)-inductively-coupled plasma mass spectrometer (ICP-MS) studies, hepta-fluorobutiric acid (HFBA), trifluoroacetic acid (TFA) and ammonium citrate from Sigma Chemical (St. Louis, MO, USA) and methanol (Sharlab, Barcelona, Spain) were used in the different chromatographic mobile phases. For the enzymatic extraction procedure the non-specific protease XIV (Sigma) was used to prepare the extracts. H_2O_2 and HNO_3 were used for acid digestion of samples. The Bradford reagent was from BioRad Laboratories S.A. All other chemicals, including glutathione reductase, reduced and oxidized glutathione, NADPH, o-phthaldehyde (OPT), dichorofluorescin (DCFH) and dinitrophenylhydrazine (DNPH) were purchased from Sigma Chemical Co. Other reagents were of analytical or chromatographic quality.

Instrumentation

For total selenium determination, samples were microwave-assisted acid digested in doubled-walled advanced composite vessels using a 1000 W MSP (Microwave Sample Preparation system) microwave oven (CEM, Matthews, NC, USA). A Sonoplus ultrasonic homogenizer (Bandelin, Germany) equipped with a titanium 3-mm-diameter microtip and fitted with an HF generator of 2200 W at a frequency of 20 KHz was used for the extraction of selenium species. An ICP-MS HP-4500 Plus (Tokyo, Japan), fitted with a Babington nebuliser and Scott double-pass spray chamber cooled by a Peltier system was used for total selenium determination after chromatographic separation. A PU-2089 HPLC pump (Jasco Corporation, Tokyo, Japan) fitted with a six-port injection valve (model 7725i, Rheodyne, Rohner Park, CA, USA) with a 100 µL injection loop was used for the chromatographic experiments.

95   Anionic exchange chromatography was performed using a Hamilton PRP-X100 (Reno,

96   NE, USA) column (10 μm particle size, 250 mm x 4.1 mm i.d.). Reversed phase

97   chromatography was performed using a C-18 Gemini column (10 μm particle size, 150

98   mm x 2.0 mm i.d) Phenomenex (Torrance, CA, USA). For molecular weight

99   fractionation, 10 kDa cut-off filters (Millipore, Bedford, MA, USA) and an Eppendorf

100   (Hamburg, Germany) centrifuge 5804, F34-6-38 were used [21].

Cell Culture

102   Human hepatoma HepG2 cells were maintained in a humidified incubator

103   containing 5 % CO_2 and 95 % air at 37ºC. They were grown in DMEM-F12 medium

104   from Biowhitaker (Innogenetics, Madrid, Spain), supplemented with 2.5 % Biowhitaker

105   foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics:

106   gentamicin, penicillin and streptomycin (all from Sigma, Madrid, Spain). Plates were

107   changed to FBS-free medium before the beginning of the assay. The serum added to the

108   medium favours growth of most cell lines but might interfere in the running of the

109   assays and affect the results. Moreover, it has been observed a fairly good growth of

110   HepG2 cells in FBS-free DMEM-F12 [17].

Cell treatment

112   Two sets of experiments were designed for this study: A) experiments of plain

113   treatment of cells with Se-MeSeCys for 2 or 20 h to test for a direct effect of the

114   selenocompound and B) experiments of pretreatment of cells with Se-MeSeCys for 2 or

115   20 h before submitting the cells to an oxidative stress by t-BOOH to test for a protective

116   effect against an oxidative insult. In order to infer the effect of the time of treatment to

117   the different concentrations of Se-MeSeCys, two experimental terms of short (2 h) and

118   long (20 h) treatment with the compound were selected according to previous studies

119   [17,18]. Lactate dehydrogenase (LDH), GSH, MDA and ROS were evaluated in both

120 experimental conditions and, in addition, GPx and GR were also determined in

121 experiment B. The different concentrations of Se-MeSeCys were dissolved in serum-

122 free culture medium. For further details see Material and Methods in references 18-20.

123 Procedure for selenium determination and speciation

124 Cultured cells were washed with phosphate buffered saline (PBS), collected by

125 scraping and resuspended in PBS. One mL of cell sample was digested in an analytical

126 microwave oven with 1 mL of nitric acid and 0.5 mL of hydrogen peroxide following

127 cycles of 5 min pressure/15 min break. Se (VI) was reduced to Se (IV) after the acid

128 digestion. Hydrochloric acid (6 M final concentration) and heating at 95ºC for 1 h are

129 needed to convert Se (VI) in Se (IV). The solutions were then diluted to 10 mL with

130 Milli-Q-water. Total selenium concentration was determined by the continuous

131 selenium hydride system connected to an AFS equipment. Hydrochloric acid (3 M) and

132 1% sodium tetrahydroborate (w/v) were used to generate the selenium hydride. For Se

133 speciation, 1 mL of cell or culture media samples, 2 mL of Milli-Q-water and 10 mg of

134 the non-specific protease *S. griseus* (protease XIV) were placed in a 10 mL Teflon vial.

135 The mixture was sonicated for 120 s at 20 W of power intensity. In order to enhance

136 sample clean-up after enzymatic hydrolysis, samples were centrifuged and the

137 supernatants were removed and passed through a 10 kDa cut-off filter (centrifugation at

138 7500 *g* and 20ºC). LC coupled with an ICP-MS was used for the measurement of

139 selenium species with the operating conditions given in Table 1.

140 Evaluation of LDH, GSH and MDA

141 Cells were plated in 60 mm diameter plates at a concentration of 1.5×10^6 per

142 plate and the assay was carried out two days later. Cells were treated as described in the

143 section above and LDH leakage to the culture medium was estimated from the ratio

144 between the LDH activity in the culture medium and that of the whole cell content

145   [16,18]. The content of GSH was quantitated by the fluorometric assay of Hissin and

146   Hilf [22]. The method takes advantage of the reaction of reduced glutathione with OPT

147   at pH 8.0. Fluorescence was measured at an emission wavelength of 460 nm and an

148   excitation wavelength of 340 nm. The precise protocol has been described elsewhere

149   [16,18]. Cellular MDA was analyzed by HPLC as its 2,4-dinitrophenylhydrazone

150   (DNPH) derivative [23]. Cells were treated as in the LDH assay and then collected. For

151   MDA, values are expressed as nmol of MDA/mg protein; protein was measured by the

152   Bradford kit.

153   **Determination of ROS**

154   Cellular reactive oxygen species were quantified by the DCFH assay using

155   microplate reader [24]. After being oxidized by intracellular oxidants, DCFH will

156   become dichorofluorescein (DCF) and emit fluorescence. By quantifying fluorescence

157   over a period of 90 min, a fair estimation of the overall oxygen species generated under

158   the different conditions was obtained. This parameter gives a very good evaluation of

159   the degree of cellular oxidative stress. The assay has been described elsewhere [16,18].

160   **Determination of GPx and GR Activity**

161   For the assay of the GPx and GR activity, cells previously treated as in LDH,

162   GSH and MDA assays were suspended in phosphate buffered saline (PBS) and

163   centrifuged at low speed for 5 min. Cell pellets were resuspended in 20 mM Tris, 5 mM

164   EDTA and 0.5 mM mercaptoethanol, sonicated, and centrifuged at 3000 g for 15 min.

165   The enzyme activity was measured in the supernatants. The determination of GPx

166   activity is based on the oxidation of reduced glutathione by GPx, using tert-butyl

167   hydroperoxide as a substrate, coupled to the disappearance of the reduced form of

168   nicotinamide adenine dinucleotide phosphate (NADPH) by GR [25]. GR activity was

169   determined by following the decrease in absorbance due to the oxidation of NADPH

170 utilized in the reduction of oxidized glutathione [26]. The methods have been previously

171 described [16,18]. Protein was measured by the Bradford kit.

172 **Statistics**

173   Statistical analysis of data was as follows: prior to analysis the data were tested

174 for homogeneity of variances by the test of Levene; for multiple comparisons, one-way

175 ANOVA was followed by a Bonferroni test when variances were homogeneous or by

176 Tamhane test when variances were not homogeneous. The level of significance was $p <$

177 0.05. A SPSS version 12.0 program has been used.

178

179 **RESULTS AND DISCUSSION**

180 **Total selenium content and speciation**

181   Analytical systems developed for the speciation of selenium species employ a

182 powerful LC coupled to a specific detector with a high efficiency sample introduction

183 system. Nowadays, the most favoured instrument combination for this purpose is ICP-

184 MS coupled to various LC techniques, such as anion-, cation-exchange

185 chromatography, reversed-phase, ion-pair, size exclusion and chiral chromatography.

186 Identification of species is achieved by retention time matching with available standards

187 utilized in a standard addition mode [21,27,28]. This analysis has not been previously

188 applied to cell culture studies.

189   Speciation of selenium was carried out in order to observe potential

190 biotransformation of Se-MeSeCys. For separation and unambiguous identification of Se

191 species two chromatographic techniques (reversed-phase chromatography and anionic

192 exchange chromatography) coupled to ICP-MS were used. The addition of a standard to

193 the sample was necessary to confirm the species that appear in chromatograms.

194 Duplicates of samples were spiked with 5 (cell samples) or 30 (culture medium

195 samples) μg/L of Se-MeSeCys at the end of the sample treatment. The single peak that

196 appears in chromatograms of cell homogenates and collected medium indicates that Se-

197 MeSeCys is the only relevant selenium species (98 % of the total selenium content) in

198 cultures of HepG2 treated with this selenium derivative, suggesting no

199 biotransformation of the compound during the length of the assay (Figure 1).

200 Preservation of the original structure of Se-MeSeCys indicates that the protective effect

201 should be bestowed to the whole molecule. Although the presence of the SH group in

202 the cysteine moiety could greatly help to increase the antioxidant potential and, thus, the

203 protective effect against an oxidative insult, the SH by itself does not necessarily confer

204 antioxidant capacity nor protection against oxidative stress to a molecule since this SH

205 group and its containing aminoacids are widely present in all proteins and most

206 biological peptides and none has shown a protective effect against an oxidative damage.

207 This fact suggests that the reducing group (SH) must be in the proper template or

208 adequate biological presentation to exert the antioxidant protective effect. Further

209 experimentation focussed separately on pure selenium and cysteine would be necessary

210 to delineate the effect of the different moieties.

211   In order to predict the potential bioavailability and metabolism of selenium and

212 its species from Se-enriched radish, Pedrero et al. [29] performed an in vitro

213 gastrointestinal process, concluding that concentration of the species found,

214 selenomethionine and Se-MeSeCys, remains almost unaltered after simulated

215 gastrointestinal digestion. Although other authors have found intense metabolism of Se-

216 MeSeCys to methyl-seleninic acid in all tissues containing beta-lyase enzyme activity

217 [30], the absence of any structural transformation in the Se-MeSeCys molecule

218 throughout the GI tract ensures preservation of the complete biological activity in a

219 strenuous metabolic environment. However, the present findings in cultured live cells

should be considered as a first necessary step in the research on the protective effects of selenium compounds against oxidative stress and future research including experiments in live animals should be delineated in order to address the in vivo metabolic fate of this compound.

Total selenium content was measured in cells treated for 24 h with Se-MeSeCys at two different concentrations (10 and 100 µM) to evaluate uptake capability of selenium by HepG2 cells. Data of selenium concentration was validated by applying the method to a certified reference material: bovine liver CRM 185R (1.68 + 0.14 mg/Kg). The result obtained, 1.8 + 0.2 mg/Kg, was in agreement with the reference value. Final concentration of Se-MeSeCys inside the cells remains relatively constant regardless of the selenium dose (13.2 ± 0.2 µg/mL homogenate in cells treated with 10 µM vs. 12.1 ± 1.1 µg/mL in those treated with 100 µM), probably due to saturation of uptake mechanisms. In fact, in human trabecular meshwork cell (HTM) treated with methylselenic acid, saturation of selenium uptake was observed at the same doses [31]. No amount of any selenium compound was found in either the culture medium or cell homogenate in HepG2 untreated cultures.

Cell viability

As a trace element, selenium appears at low concentrations in the diet but chemopreventive (antioxidant and antitumorigenic) levels of selenium appear to reside at doses greater than those regarded as necessary for supporting adequate expression of selenoenzymes [32,33]. Selenium requirements in human diet are in a very narrow range: consumption of food containing less than 0.1 mg kg^{-1} of this element will result in its deficiency, whereas dietary levels above 1 mg kg^{-1} will lead to toxic manifestations [34,35]. Although selenite has been reported to protect human endothelial cells from oxidative damage induced by a potent prooxidant [36], it has

245 been suggested that the cancer chemopreventive effect of Se-MeSeCys in tumor cells

246 may reflect pro-oxidant rather than antioxidant activity of this compound [14].

247 Therefore, before aiming for the protective effect of the tested antioxidant it is necessary

248 to ensure that no direct damage is caused to the cell by the compound. Thus, in

249 experiment A, cell toxicity and basal cellular redox status were determined in cells

250 treated for short (2 hours) and long (20 h) terms with different concentrations of Se-

251 MeSeCys in the nM-μM range. The concentration range is fairly realistic in order to

252 evaluate the effect at the physiological level since steady-state concentrations around

253 0.7 μM of all selenium species have been reported in human serum [37]. Our results

254 show that treatment with concentrations of Se-MeSeCys up to 10 μM for 20 h evoked

255 no toxicity in HepG2 (Figure 2a). Indeed, the complete inhibition of cell damage in

256 experiment B when human hepatoma HepG2 cells were pretreated with Se-MeSeCys

257 for 2 or 20 h prior to being submitted to t-BOOH (Figure 3a), indicates that integrity of

258 the Se-MeSeCys-treated cells was largely protected against the oxidative insult.

259 **GSH concentration**

260 Cells are naturally provided with an extensive array of protective enzymatic and

261 non-enzymatic antioxidants that counteract the potentially injurious oxidizing agents

262 [2,16-20,32,36]. But even this multifunctional protective system cannot completely

263 prevent the deleterious effects of ROS, and consequently oxidatively damaged

264 molecules accumulate in cells. Reduced glutathione is the main non-enzymatic

265 antioxidant defense within the cell and plays an important role in protection against

266 oxidative stress, as a substrate in glutathione peroxidase-catalysed detoxification of

267 organic peroxides, by reacting with free radicals and by repairing free-radical-induced

268 damage through electron-transfer reactions [2,16-20]. It is usually assumed that GSH

269 depletion reflects intracellular oxidation. On the contrary, an increase in GSH

270 concentration could be expected to prepare the cell against a potential oxidative insult

271 [16-20,38,39].

272   In our experimental conditions, addition of Se-MeSeCys did not evoke changes

273 in GSH concentration whereas treatment of HepG2 cells with 200 μM t-BOOH induced

274 a remarkable decrease in the concentration of reduced glutathione indicative of

275 oxidative stress (Figure 2b). This decrease of GSH induced by t-BOOH was partly (2 h)

276 or completely (20 h) prevented by pretreatment with Se-MeSeCys (Figure 3b). This

277 result could explain the protected cell integrity reported above since maintaining GSH

278 concentration above a critical threshold while facing a stressful situation represents an

279 advantage for cell survival.

280 **MDA concentration**

281   An important step in the degradation of cell membranes is the reaction of ROS

282 with the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid

283 hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can

284 be formed; MDA, a three-carbon compound formed by scission of peroxidized PUFAs,

285 mainly arachidonic acid, is one of the main products of lipid peroxidation [40]. Since

286 MDA has been found elevated in various diseases thought to be related to free radical

287 damage, it has been widely used as an index of lipoperoxidation in biological and

288 medical sciences [41]. However, other than our previous results, determination of MDA

289 levels in cell culture conditions is extremely scant in the literature [42].

290   We have established a new method of evaluation of MDA in cultures of human

291 hepatoma cells that is sensitive enough to detect a significant increase in MDA

292 concentration in response to an oxidative stress induced by t-BOOH [23]. By using this

293 method we have found that a 3 h treatment of HepG2 with 200 μM t-BOOH evoked a

294 significant increase of about 35 % in the cellular concentration of MDA, indicating

295   damage to cell lipids (Figures 2c and 3c). In fact, a significant decrease of MDA was

296   observed in cells treated with 1 or 10 μM Se-MeSeCys for 20 h in experiment A (Figure

297   2c). In addition, the t-BOOH-induced increase of MDA was completely avoided when

298   cells were pretreated for 2 or 20 h with 0.1-10 μM of Se-MeSeCys in experiment B

299   (Figure 3c). This protection by Se-MeSeCys against an induced lipid peroxidation in a

300   cell culture has not been previously reported and is in line with previous studies that

301   showed a similar effect by other dietary compounds including plant polyphenols such as

302   tea catechins [43,44], quercetin [18] and olive oil hydroxytyrosol [19], beta carotene or

303   lutein [43], and Maillard reaction products such as coffee melanoidin [20] in the same

304   cell line, human hepatoma HepG2.

305   **ROS Generation**

306   Accumulation of ROS in several cellular components is thought to be a major

307   cause of molecular injury leading to cell aging and to age-related degenerative diseases

308   such as cancer, brain dysfunction and coronary heart disease [2,3,32,36]. Direct

309   evaluation of ROS yields a very good indication of the oxidative damage to living cells

310   [24]. Based upon the fact that nonfluorescent DCFH crosses cell membranes and is

311   oxidized by intracellular ROS to highly fluorescent DCF [45], the intracellular DCF

312   fluorescence can be used as an index to quantify the overall oxidative stress in cells [16-

313   20]. A prooxidant such as t-BOOH can directly oxidize DCFH to fluorescent DCF, and

314   it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus

315   increasing fluorescence.

316   A significant increase in ROS generation was observed in HepG2 cells treated

317   with 200 μM t-BOOH for the 90 min of the assay, whereas ROS levels in cells treated

318   with 0.01-10 μM Se-MeSeCys in experiment A were below those of control non-

319   stressed cells (Figure 4). Similar to what observed with other competent antioxidants

320   [18,19], the presence of Se-MeSeCys in the cell culture appears to reduce the

321   progressive formation and accumulation of oxygen radicals by the mitochondrial

322   respiratory chain [18,19]. The increased ROS generation induced by t-BOOH was not

323   prevented in cultured cells pretreated for 2 or 20 h with Se-MeSeCys in experiment B

324   (Figure 5), contrary to a previous report with quercetin [18], but similar to what

325   observed with other dietary antioxidants such as the olive oil phenol hydroxytirosol [19]

326   and coffee melanoidin [20]. In the 2 h pre-treatment condition, this phenomenon is

327   consistent with the partial recovery of the levels of reduced glutathione which may be

328   explained by an increased consumption of GSH in the enzymatic and non-enzymatic

329   quenching of reactive oxygen species generated by t-BOOH. In any case, these data

330   suggest that high levels of ROS generated during the stress period are being more

331   efficiently quenched in cells pretreated with Se-MeSeCys resulting in a reduced cell

332   damage and lipid peroxidation.

333   **GPx and GR activity**

334   In the defense against oxidative stress, the cellular antioxidant enzyme system

335   plays a crucial role and changes in the activity of antioxidant enzymes can be

336   considered as biomarkers of the antioxidant response [16-20,46,47]. GPx catalyses GSH

337   oxidation to GSSG at the expense of H_2O_2 or other peroxides [47] and GR recycles

338   oxidized glutathione back to reduced glutathione [3,26], therefore, their activities are

339   essential for the intracellular quenching of cell-damaging peroxide species and the

340   effective recovery of the steady-state concentration of reduced glutathione.

341   The chemopreventive activity of selenium has been attributed to its effect on a

342   variety of molecular targets and cellular processes, although it appears that these targets

343   and processes may differ with the prooxidant or tumorigenic agent and the chemical

344   nature of the selenium compound [1,2,6,32,36]. Nevertheless, linkage of the antioxidant

345 and anti-tumorigenic activity with positive effects on "protective" enzymes remains a

346 recurrent theme and a common feature of most of the implicated enzymes is their

347 function in sequestering reactive oxygen species and/or maintaining the cell and cellular

348 components in their appropriate redox state [1,2,32,33,36]. Some protective enzymes

349 such as GPx are selenoproteins and are likely to be impacted by selenium

350 supplementation [1,36]. The significant increase in the activity of GPx and GR observed

351 after a 3 h treatment with 200 μM t-BOOH (Figure 6), clearly indicates a positive

352 response of the cell defense system to face an oxidative insult [16-20]. Other dietary

353 antioxidants have been tested by other authors and significant changes in the enzyme

354 activity of the antioxidant enzymes have been observed only at very high doses [3,48].

355 Although only a previous cell culture study has shown a specific effect of a selenium

356 compound on the GPx response to an oxidative insult [36], in experimental conditions

357 similar to those of the present study we have shown that the flavonoid quercetin [18],

358 olive oil phenol hydroxytyrosol [19] and a coffee melanoidin [20] protect cell damage

359 by preventing the severely increased activity of antioxidant enzymes induced by t-

360 BOOH. In line with those results, in experiment B of the present study we show, for the

361 first time, that a long-term treatment of human hepatoma cells with Se-MeSeCys

362 prevents the increase in the activity of GPx and GR induced by oxidative stress (Figure

363 6).

364

365 **CONCLUSIONS**

366   The results indicate that at the end of an induced stress period the antioxidant

367 defense system of cells that had been pretreated with Se-MeSeCys has more efficiently

368 returned to a steady-state activity diminishing, therefore, cell damage and enabling the

369 cell to cope in better conditions with further oxidative insults.

370   In addition, our results support previous data on the chemoprotective effect of

371   Se-MeSeCys and give more insight on its potential biological activity, showing that

372   concentrations of Se-MeSeCys within the physiological range remain unaltered during

373   the treatment and evoke a favourable response in cellular models. Therefore, Se-

374   MeSeCys may contribute to the protection afforded by fruits, vegetables and plant-

375   derived beverages against diseases for which excess production of ROS has been

376   implicated as a casual or contributory factor.

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FIGURE CAPTIONS

Figure 1.- Chromatogram of Se species by a) anionic exchange chromatography in cell homogenates, b) reversed-phase chromatography in cell homogenates and c) anionic exchange chromatography in collected media from cell cultures.

Figure 2.- Experiment A. Effect of Se-MeSeCys on cell viability and intracellular concentration of GSH and MDA. Cells treated with 200 μM t-BOOH for 3 h (noted t-BOOH) are positive controls. a) LDH values are means ± SD of 6-8 data. b) GSH values are means of 4-5 different samples per condition. c) MDA values are means ± SD, n= 4. Groups of data within the same graphic with a different letter are statistically different ($P < 0.05$).

Figure 3.- Experiment B. Protective effect of Se-MeSeCys on cell viability and intracellular concentration of GSH and MDA. a) LDH values are means ± SD of 6-8 data. b) GSH values are means of 4-5 different samples per condition. c) MDA values are means ± SD, n= 4. Groups of data within the same graphic with a different letter are statistically different ($P < 0.05$).

Figure 4.- Experiment A. Effect of Se-MeSeCys on intracellular ROS generation. Cells treated with 200 μM t-BOOH for 3 h (noted t-BOOH) are positive controls. Different letters upon symbols indicate statistically significant differences ($P < 0.05$) when that group or close groups of data are compared within the same time point. Values are

means ± SD of 7-8 different samples per condition. SD values were not included due to intense bar overlapping.

Figure 5.- <u>Experiment B.</u> Protective effect of Se-MeSeCys on intracellular ROS generation. Different letters upon symbols indicate statistically significant differences ($P < 0.05$) when that group or close groups of data are compared within the same time point. Values are means ± SD of 7-8 different samples per condition. SD values were not included due to intense bar overlapping.

Figure 6.- <u>Experiment B.</u> Protective effect of Se-MeSeCys on the activity of GPx and GR. Groups of data within the same graphic with a different letter are statistically different ($P < 0.05$). Values are means ± SD of 4-5 different samples per condition.

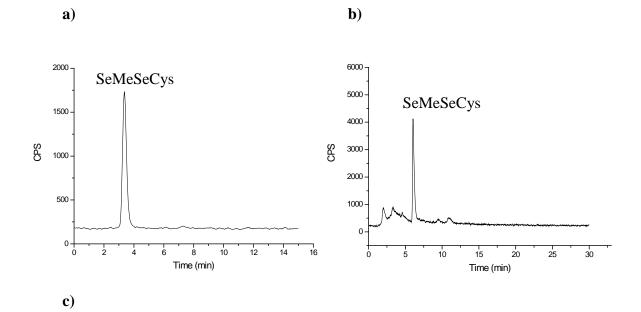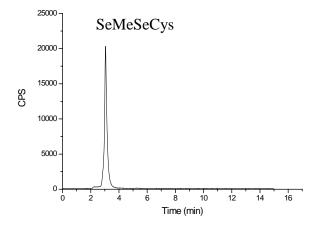**Table 1** Instrumental operating conditions for Se determination by HPLC-ICP-MS

HPLC parameters

Analytical column	PRP-X100	Phenomenex C-18
Eluent	10mM citrate buffer, H_2O:MeOH (98:2)	0.1% HFBA; 0.05% TFA; 2% MeOH
Eluent flow rate	1 ml min^{-1}	0.2 ml min^{-1}
Elution programme	Isocratic	Isocratic
Injection volume	100 µl	20 µl

ICP-MS conditions

Forward power	1350 W
Plasma gas (Ar) flow rate	15 l min^{-1}
Auxiliary gas (Ar) flow rate	1.3 l min^{-1}
Carrier gas (Ar) flow rate	1.4 l min^{-1}
Nebulyser type	Babington
Isotopes monitored	^{77}Se, ^{78}Se, ^{82}Se

Figure 1

a)

b)

c)

Figure 2

a)

b)

□ 2 h
■ 20 h

c)

Figure 3

a)

b)

c)

27

Figure 4

a)

2 h

b)

20 h

Figure 5

a)

2 h

b)

20 h

Figure 6

a)

b)