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Cytoplasmic and nuclear toxicity of 3,5-dimethylaminophenol and potential protection by selenocompounds

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ABSTRACT

Most common alkylanilines in the environment are 2,6-dimethylaniline (2,6-DMA), 3,5-dimethylaniline (3,5-DMA), and 3-ethylaniline (3-EA). 3,5-Dimethylaminophenol (3,5-DMAP), a metabolite of 3,5-DMA, is of particular interest, as it is potentially genotoxic. Supplementation with organic or inorganic forms of selenium (Se) may reduce toxicity following exposure to a wide variety of environmental chemicals. This study was designed to evaluate the protective effects of sodium selenite (SS) and selenomethionine (SM) at varying time points of supplementation (24 h and 72 h) against the cytotoxicity, reactive oxygen species (ROS) production, and genotoxicity of 3,5-DMAP in CHO AS52 cells. 3,5-DMAP caused dose-dependent increase of cytotoxicity, ROS production and genotoxicity, and generated free radicals in the nuclei. Thioredoxin reductase (TrxR), catalase and glutathione reductase activities, and glutathione levels were significantly lower while lipid peroxidation and protein oxidation levels were higher after 3,5-DMAP treatment in both cytoplasm and the nucleus vs. control. After 24 h, both SS and SM provided protection in antioxidant/oxidant status of the 3,5-DMAP-treated cells; however other than supplying higher glutathione peroxidase and TrxR activities, 72 h supplementation did not provide advanced improvement. Selenocompounds may be beneficial against cytotoxic and genotoxic potential of 3,5-DMAP and might protect both nucleus and cytoplasm following exposure to alkylanilines.

1. Introduction

Oxidative stress can account for changes that are detrimental to cells. Reactive oxygen species (ROS) are shown to contribute to cellular damage, apoptosis or carcinogenesis (Dalton et al., 1999; Dröge, 2002; Reuter et al., 2010). Currently, there is a growing interest in environmental chemicals that can induce ROS, like alkylanilines (Soory, 2009).

Epidemiological studies have demonstrated that alkylanilines are a group of chemicals that are ubiquitous in the environment. Most individuals experience lifelong exposure to these compounds from occupational exposure or *via* tobacco smoke (Skipper et al., 2010). They are considered factors for the development of bladder cancer (Skipper et al., 2010). Alkylanilines are potentially activated through the P450-catalyzed oxidation of the amino group, subsequently undergoing oxidation of the *N*-hydroxylamine and heterolysis of the N–O bond to produce a reactive nitrenium ion (Chao et al., 2012). The *N*-hydroxylation product of arylamines could form para-*N*-phenyl-hydroxylamine (DMHA) and is isomeric

Abbreviations: 2,6-DMA, 2,6-dimethylaniline; 3,5-DMA, 3,5-dimethylaniline; 3,5-DMAP, 3,5-dimethylaminophenol; 3,5-DMIQ, 4-amino-3,5-dimethylphenol iminequinone; 3-EA, 3-ethylaniline; 6-TG, 6-thioguanine; BCA, bicinchoninic acid assay; CAT, catalase; CHO, Chinese Hamster Ovary cells; CM-H_2DCFA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DMHA, para-*N*-phenyl-hydroxylamine; DMSO, dimethyl sulfoxide; DPNH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EMS, ethyl methansulfonate; ESI-TOF-MS, electrospray ionization source time-of-flight and tandem quadrupole mass spectrometry; FBS, fetal bovine serum; *gpt*, xanthine–guanine phosphoribosyl transferase gene; GPxs, glutathione peroxidases; GSH, reduced glutathione; GSSG, oxidized glutathione; HBSS, Hank's Buffered Salt Solution; HNE, 4-hydroxynonenal; HPRT, hypoxanthine–guanine phosphoribosyl transferase; LC, liquid chromatography; MAAs, monocyclic aromatic amines; MDA, malonyldialdehyde; NAC, N-acetylcysteine; NADP, Nicotinamide adenine dinucleotide phosphate; NE-PER, nuclear and cytoplasmic extraction kit; OTM, olive tail moment; p-AP, para-aminophenol; ROS, reactive oxygen species; Se, selenium; SM, selenomethionine; SOD, total superoxide dismutase; SS, sodium selenite; TBARS, thiobarbituric acid reactive substance; TNB, 5-thio-2-nitrobenzoic acid; TrxRs, thioredoxin reductases; XO, xanthine oxidase; XPRT, xanthine-guanine phosphoribosyl transferase.

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with para-aminophenol (p-AP). The hydroxylamine might also be a source of DMHA, which is known to rearrange to DMAP under mildly acidic conditions (Sone et al., 1981; Fishbein and McClelland, 1987). The mutagenic activity of p-AP was suppressed with the addition of dimethylsulfoxide (DMSO) or catalase (CAT), suggesting the involvement of ROS in the mutagenic activity of p-AP (Yoshida et al., 1998).

3,5-Dimethylaminophenol (3,5-DMAP), a metabolite of 3,5-DMA, is of particular interest, as it is potentially genotoxic by a mechanism involving non-enzymatic oxidation to the quinoneimine and quinone (Jefferies et al., 1998; Yoshida et al., 1998; Ye et al., 2012). Chao et al. (2014) showed that 3,5-DMAP caused apoptosis *via* generation of H_2O_2 and hydroxyl radical (·OH) and upregulation of caspase 3 in Chinese Hamster Ovary (CHO) AS52 cells (Ye et al., 2012; Chao et al., 2014). Therefore, it can be suggested that one of the major causes underlying the toxicity of 3,5-DMAP is oxidative stress.

There is considerable interest in developing strategies to prevent the cytotoxicity and genotoxicity induced by alkylanilines and their metabolites, with minimal risk or toxicity. If not counteracted by cellular antioxidants, high levels of ROS can cause acute injury and damage of important biomolecules including cellular proteins, lipids and DNA and this phenomenon may lead to cell death (Martindale and Holbrook, 2002; Kern and Kehrer, 2005). Published data associates both low antioxidant status and genetics as contributing factors to the risk of several types of malignancies (Baliga et al., 2007; Lee, 2009). Trace elements like selenium (Se) are a key component of several antioxidant enzyme systems. Se-dependent glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) protect the body from cellular metabolism's endogenous by-products, which are associated with DNA damage, mutagenesis, and carcinogenesis (Ganther, 1999; Jablonska et al., 2009). Se was found to be protective against the toxic potential of different agents both *in vitro* and *in vivo* (Erkekoglu et al., 2010a, 2010b;Sharma et al., 2014), through different pathways some of which need to be discovered.

Research has shown that low, non-toxic supplementation with either organic [selenomethionine (SM)] or inorganic [sodium selenite (SS)] selenium forms can reduce cancer incidence following exposure to a wide variety of carcinogens (el-Bayoumy et al., 1991). Se supplementation was shown to reduce the risk of many types of neoplasia, including bladder cancer. The results from seven epidemiological studies conducted in different populations indicate that high levels of serum Se provided a significant and 39% decreased risk of bladder cancer (Amaral et al., 2010).

Based on this background information and taking into account the importance of Se in the antioxidant system, this study was designed to evaluate the protection offered by supplementation with different selenocompounds, SS and SM, at varying time points of exposure against 3,5-DMAP-caused cytotoxicity, ROS production, and genotoxicity in CHO AS52 cells.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals including SS, SM, 6-thioguanine (6-TG), dimethyl sulfoxide (DMSO), ethyl methansulfonate (EMS), DL-isocitric acid, triethanolamine, vinylpyridine, protease inhibitor cocktail were purchased from Sigma–Aldrich (St. Louis, MO). Nicotinamide adenine dinucleotide phosphate (NADP) was purchased from Boehringer Mannheim (Indianapolis, IN). Cell proliferation reagent WST-1 from Roche Applied Science (Indianapolis, IN). 5-(and 6-)Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2 DCFA) ROS dye, molten normal melting point agarose, low melting point agarose, SYBR Gold and Hoechst 33258 were obtained from Molecular Probes/Invitrogen (Eugene, OR).

2.2. Kits

Colorimetric assay kit for bicinchoninic acid (BCA) assay, and spectrophotometric GPx1, TrxR, CAT, total superoxide dismutase (SOD), glutathione reductase (GR), glutathione S-transferase (GST), glutathione (GSH), caspase 3 and caspase 8 kits were obtained from Sigma–Aldrich (St. Louis, MO). Nuclear and Cytoplasmic Extraction kit (NE-PER) were purchased from Thermo Scientific (Rockford, IL). Thiobarbituric acid reactive substance (TBARS) kit and carbonyl assay kit were obtained from Cayman Chemical Company (Ann Arbor, MI). All cell culture reagents and GelBond film were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Bottomless 96-well plates for Comet assay were purchased from Greiner BioOne (Monroe, NC).

2.3. Synthesis of 3,5-dimethylaminophenol

2.3.1. Instrumentation

NMR spectra were recorded on a Varian INOVA NMR spectrometer (Varian, Inc., Palo Alto, CA) at 500 MHz for ^{1}H NMR. Analyses were performed in high resolution detection on an electrospray ionization source time-of-flight and tandem quadrupole mass spectrometry (ESI-TOF-MS) (Agilent, Palo Alto, CA). ESI was conducted using a needle voltage of 3.5 kV. Nitrogen was used as the drying (12 L/min) and nebulizer (35 psig) gas with the heated capillary at 325 °C. The ion trap mass spectrometer was operated in full scan. Chromatography was performed using an Agilent 1200 LC system. Liquid chromatography (LC) separations were performed using a Zorbax Eclipse XDB-C18 (2.1 mm × 150 mm; 5 μm; Agilent column with a flow rate of 0.3 mL/min). Solvent A was 0.1% (h/h) formic acid in water, and solvent B was 0.1% (h/h) formic acid in methanol.

2.3.2. 3,5-Dimethylaminophenol

3,5-DMAP was synthesized according to Chao et al. (2012). The yield of final product was 82%, the positive ion in high resolution detection by performed by ESI-TOF-MS: calculated for $[C_8H_{11}NO]H^+$, 138.0913, found 138.0910. ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): 7.17 (s, 1H), 6.15 (m, 2H), 4.3 (s, 2H), 2.02 (s, ^{6}H).

2.4. 4-Amino-3,5-dimethyl quinoneimine (3,5-DMQI) synthesis

The 3,5-DMAP (35 mg) was dissolved in 10 mL of ethyl acetate and mixture was stirred under argon atmosphere. Lead dioxide (350 mg) was added and was stirred for 30 min. The reaction mixture was washed with 10 mL of 5% aqueous $NaHCO_3$. The high resolution detection was performed by ESI-TOF-MS and was calculated for $C_8H_9NO]H^+$, 136.0757 and was found 136.0751. The results for ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) were 11.59 (s, 1H, OH), 7.05 (s, ^{1}H, Ar–H), 6.87 (s, ^{1}H, Ar–H), 1.94 (s, ^{3}H, Methyl-H), 1.89 (s, ^{3}H, Methyl-H).

2.5. Determination of the conversion rate of 3,5-dimethylaminophenol to 4-amino-3,5-quinoneimine

Cell media including 3,5-DMAP (50 μM) was analyzed by liquid chromatography-ESI-TOF-MS (LC-ESI-TOF-MS) with or without cells at 0 h and 1 h. Media were analyzed by ESI-TOF-MS. The full scans of each sample were extracted with exact mass of the 3,5-DMAP. The consumption rate of the 3,5-DMAP was also measured for 1 h.

2.6. Determination of the interaction of 3,5-dimethylaminophenol with sodium selenite and selenomethionine

One mL of 10 mM SS or 1 mL of 10 mM SM solution (mixture of 50% methanol:water) was added to the 3,5-DMQI solution (~0.01 mmol) with stirring. After 1 h incubation at 25 °C, the reaction solution was analyzed by LC-ESI-TOF-MS for 20 min.

2.7. Cell culture and treatment

AS52 cells used in the study were kindly provided by Dr. Helga Stopper (University of Würzburg, Germany). These cells are transformants constructed from a hypoxanthine–guanine phosphoribosyl transferase (HPRT)-deficient CHO cell line and contain a single, functional copy of the Escherichia coli guanine phosphoribosyl transferase gene (*gpt*) stably integrated into the Chinese hamster genome. AS52 cells were chosen according to the results of our previous experiments which showed that these cells were susceptible to the toxic effects of 3,5-DMAP at relatively low doses compared to human primary bladder cell lines (Chao et al., 2012; 2014).

Cells were cultured in Ham's F-12 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FBS (complete medium) in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was changed every 3 days. When the cell confluency reached ~90%, the cells were subcultured with trypsinization. Cells were cleansed of preexisting *gpt* mutants for genotoxicity studies by culturing in MPA medium (10 μg/mL MPA, 250 μg/mL xanthine, 22 μg/mL adenine, 11 μg/mL thymidine and 1.2 μg/mL aminopterin) for 7 days. Cells were then passed to the recovery medium enriched with xanthine (11.5 μg/mL), adenine (3 μg/mL) and thymidine (1.2 μg/mL) for another 3 days.

2.8. Experimental groups

AS52 cells (1×10^6/dish) were plated in 6-well plates one day prior to treatment and two sets of cells were prepared: 24 h SS or SM groups and 72 h SS and SM groups. The groups that received Se supplementation were incubated in media with SS or SM for 24 h or for 72 h at 37 °C. The 3,5-DMAP groups were treated with 3,5-DMAP for 1 h at 37 °C. Cells were washed twice with serum free medium and incubated in complete medium for 24 h at 37 °C. Throughout the figures and tables, results obtained only from 24 h groups are presented as 24 h Se supplementation was found to be as protective as 72 h Se supplementation.

The experimental groups were:

(1) Control 24 Group *(Control)*: Control AS52 cells.
(2) SS 24 h Group *(SS)*: AS52 cells pre-treated with SS (30 nM) for 24 h.
(3) SM 24 h Group *(SM)*: AS52 cells pre-treated with SM (10 μM) for 24 h.
(4) DMAP 24 h group *(DMAP)*: AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h.
(5) SS DMAP 24 h group *(SS/DMAP)*: AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. and cultured for 1 h.
(6) SM DMAP 24 h group *(SM/DMAP)*: AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h.

2.9. Preparation of nuclear and cytoplasmic extracts

Cells were harvested and centrifuged at 500*g* for 5 min after treatments. The pellets were washed with PBS and the supernatant was discarded. The rest of the pellet was used to prepare nuclear and cytoplasmic extracts using NE-PER kit in the presence of protease inhibitor cocktail.

2.10. Determination of cytotoxicity

Cytotoxicity was determined using WST-1 assay. After treatments, cells were trypsinized and suspended in 1 mL complete medium. 10 μL of the cell suspensions were pipetted into a 96-well plates. A mixture of 10 μL water-soluble WST-1 kit reagent plus 80 μL medium was added to each well of the 96-well plate. The plate was incubated at 37 °C for 1 h in the dark and absorbance was measured at 495 nm with the μQuant micro-reader (Biotek Instruments Inc. Winooski, VT). The values were normalized and presented as the percentage of the control.

2.11. Quantification of intracellular reactive oxygen species production

Intracellular ROS detection studies were performed using CM-H_2DCFDA ROS detection assay. Cells were placed in 6 well plates at the same density of cytotoxicity assay as described above. After the treatment, cells were washed with PBS, trypsinized and resuspended in 1 mL serum free medium/well. Cell suspensions (100 μL) were pipetted into 96-well plate and mixed with 10 μL Hank's Buffered Salt Solution (HBSS) containing CM-H_2DCFDA (25 μM, preincubated at 37 °C for 30 min). ROS generation was measured immediately at an excitation wavelength of 485 nm and emission wavelength of 530 nm, by using HTS 7000 Plus Bio Assay microreader (Perkin Elmer Life Sciences, Waltham, MA). The ROS production values were normalized to the control and presented as percentage of control.

2.12. Nuclear staining for 3,5-dimethylaminophenol

Nuclear staining for ROS production was performed using a CM-H_2DCFDA dye. After 1 h of 3,5-DMAP exposure, cells were washed as mentioned above. Cytoplasmic and nuclear extracts were prepared using NE-PER kit. The nuclear pellet (10 μL) was mixed with 1 μL HBSS containing CM-H_2DCFDA (25 μM) and activated at 37 °C for 30 min. Nuclei were stained with Hoechst 33258 (1 μg/mL). The CM-H_2DCFDA-labeled nuclear pellets were place on a glass slide and visualized by Nikon epifluorescence microscope (Tokyo, Japan) at 400× magnification (excitation wavelength 488 nm).

2.13. Determination of antioxidant enzyme activities

Throughout the spectrophotometric and spectrofluorometric measurements, SpectraMax M2 (Molecular Devices, Sunnyvale, CA) was used as spectrophotometer and spectrofluorometer. SoftMax Pro software (Molecular Devices, Sunnyvale, CA) was used for quantification.

The activity of GPx1 was measured in a coupled reaction with glutathione reductase (GR) as described earlier using a "glutathione peroxidase assay kit" (Günzler et al., 1974; Flohé and Günzler, 1984). 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 reduced glutathione (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 of GPx1 that transformed 1 μmole of NADPH to NADP per min at 37 °C.

TrxR activity was determined using the "thioredoxin reductase assay kit". As described previously (Arnér et al., 1999), the method was based on the reduction of DTNB (in the presence of NADPH) to 5-thio-2-nitrobenzoic acid (TNB), the concentration of which was measured at 412 nm. One unit of TrxR activity was defined as the

amount of enzyme that caused an increase absorbance of 1.0 per min and per mL at pH 7.0 at 25 °C.

Activity of CAT was determined colorimetrically using a "catalase assay kit". The enzymatic decomposition of H_2O_2 was stopped with a stop solution and the remaining H_2O_2 was detected with a chromogen solution (150 mM potassium phosphate buffer, pH 7.0,containing 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid) at 520 nm. One unit of catalase was defined as the enzyme activity that decomposed one μM of H_2O_2 to oxygen and water per min at pH 7.0 and 25 °C.

The total SOD activity was measured by a "total superoxide dismutase activity kit" colorimetrically. The activity was determined by using WST-1 that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 was linearly related to the xanthine oxidase (XO) activity and was inhibited by SOD. The IC_{50} (50% inhibition activity of SOD) was determined by this colorimetric method. Since the absorbance at 440 nm was proportional to the amount of superoxide anion, the inhibition of SOD activity was quantified by measuring the decrease in the color development at 440 nm.

The activity of GR was measured by a "glutathione reductase assay kit", based on the reduction of oxidized glutathione (GSSG) by GR in the presence of NADPH. One unit of enzyme was defined as the enzyme activity that caused the reduction of 1 μmol of DTNB at 25 °C (Goldberg and Spooner, 1983). The results are expressed in mU/mg protein.

GST activity was determined using a "glutathione S-transferase assay kit". 1-chloro-2,4 dinitrobenzene (CDNB) was used as a substrate, monitoring the rate of production of 2,4-diphenyl glutathione at 340 nm and the enzyme activity was expressed in nmol/mg protein/min (Habig et al., 1974).

2.14. Determination of total, reduced and oxidized glutathione levels

Total glutathione content of the cell extracts was assessed using a "total glutathione assay kit" based on a kinetic assay in which catalytic amounts of GSH caused a continuous reduction of DTNB to TNB (Akerboom and Sies, 1981). For oxidized glutathione (glutathione disulfide, GSSG) determination, GSH was inactivated by the addition of 2-vinylpyridine in the presence of triethanolamine. Quantification was achieved by parallel measurements of a standard curve of known GSH or GSSG concentrations, and results were expressed in nmol/mg protein. GSH content was calculated using the equation of GSH = (Total glutathione $- 2 \times$ GSSG). GSH/GSSG redox ratio was calculated by dividing the GSH values to GSSG values.

2.15. Determination of lipid leroxidation

Lipid peroxidation (LP) in nuclear and cytoplasmic extracts was quantified measuring the concentration of TBARS by a spectrofluorometric assay using a "TBARS assay kit" as described by Richard et al. (1992) (Richard et al., 1992). Quantification was achieved by parallel measurements of a standard curve of known TBARS concentrations, and results were expressed as nmol/g protein.

2.16. Determination of protein oxidation

The carbonyl groups as the biomarker of protein oxidation were determined by using 2,4-dinitrophenylhydrazine (DPNH) reaction and the amount of protein-hydrazone produced was quantified spectrophotometrically at 360 nm using a "carbonyl assay kit".

2.17. Determination of caspase 3 and caspase 8 activities

The caspase 3 activity was measured by using a "caspase 3 colorimetric assay kit". The caspase 3 assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

The caspase 8 activity was measured by using a "caspase 8 colorimetric assay kit". The assay is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp *p*-nitroaniline (Ac-IETD-pNA) by caspase 8 resulting in the release of a pNA moiety. The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve performed with defined pNA solutions.

2.18. Protein determination

Protein content of the samples was determined by BCA using a "protein assay kit". The results were obtained as mg/mL protein (Krieg et al., 2005).

2.19. CometChip and alkaline Comet assay

The alkaline comet assay was performed on the CometChip using the protocol described by Wood et al. (2010). Molten 1% normal melting point agarose was poured on top of a sheet of GelBond film and the polydimethylsiloxane (PDMS) mold with microposts were floated until the agarose cool and gel. After the mold was removed, the agarose gel (with microwells that were attached to GelBond film) was placed on a glass plate, a bottomless 96-well plate was pressed onto the gel and clamped to create the multi-well version of the comet platform, the CometChip.

After the treatment as mentioned above, 100 μL of cells (10^6 cells/ml) were pipetted into each of the 96-well and captured in microwells by gravity. The bottomless 96-well plate was then removed and the gel was covered with 1% low melting point agarose. After overnight lysis, the CometChips were placed into an electrophoresis chamber filled with alkaline unwinding buffer (0.3 M NaOH and 1 mM Na_2EDTA) for 40 min at 4 °C. Electrophoresis was performed at the same temperature with the same buffer for 30 min at 1 V/cm and a current of 300 mA. The chips were then neutralized twice for 15 min in fresh buffer (0.4 M Tris–HCl at pH 7.5) at 4 °C. After neutralization, the CometChips were stained with SYBR Gold according to the manufacturer's instructions for the fluorescence imaging. Images were captured using a Nikon 80i upright microscope (Tokyo, Japan) coupled with an automatic scanning stage and analyzed using the "Guicometanalyzer", a custom software written in MATLAB (The Mathworks, Natick, MA). The results generated by the software showed percentage of tail DNA which represented as the level of DNA damage and olive tail moment (OTM, product of comet length and tail intensity). The cells treated with 100 μM H_2O_2 were used as the positive control and cells treated with 1% DMSO were used as negative control.

2.20. Statistical analysis

All the experiments were done in triplicate. The results were expressed as mean ± standard deviation (SD). The differences among the groups were evaluated with ANOVA, followed by Mann–Whitney U test using a Statistical Package for Social Sciences Program (SPSS) version 17.0. p-values < 0.05 were considered as statistically significant.

3. Results

3.1. 3,5-Dimethylaminophenol and 3,5-dimethyaminolphenol quinoneimine synthesis

^{1}H NMR of 3,5-DMAP and ^{1}H NMR of 3,5-DMQI are shown in Supplementary Data (Fig. A and B).

3.2. Conversion rate from 3,5-dimethylaminophenol to 4-amino-3,5-dimethylphenol quinoneimine

The conversion rates of 3,5-DMAP to 3,5-DMQI in the media with or without cells were ~99.4% and ~98.7% in 1 h, respectively (Supplementary Data Fig. C).

3.3. Interaction of 4-amino-3,5-dimethyl quinoneimine with sodium selenite and selenomethionine

SS and its oxidized form could not be ionizated both in positive or negative mode and 3,5-DMQI was still kept in the quinoneimine form after 1 h incubation (SS+3,5-DMQI) (Fig. 1). Oxidation scheme of SM after 1 h incubation with 3,5-DMQI is shown in Fig. 2. Total ion chromatograms of SM and 1 h incubation of SM with 3,5-DMQI are given Fig. 3A and B. 40% of SM was oxidized to methionine selenoxide and 3,5-DMQI was still kept in the iminequinone form after 1 h incubation (SM+3,5-DMQI). Therefore, no direct reduction of 3,5-DMAP was observed with selenocompounds.

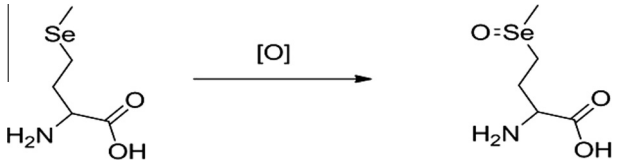

Fig. 2. Oxidation scheme of SM after 1 h incubation of SM with 3,5-DMQI. 3,5-DMIQ; 4-amino-3,5-dimethylphenol iminequinone; SM, selenomethionine.

3.4. 3,5-Dimethylaminophenol-induced ROS production and cytotoxicity and possible protection by selenocompounds

As previously observed, 3,5-DMAP caused a dose-dependent increase of cytotoxicity in the AS52 cells (Ye et al., 2012; Chao et al., 2014). The half maximal inhibitory concentration (IC_{50}) of 3,5-DMAP was 25 μM. In Fig. 4, pretreatments with SS and SM for 24 h was not cytotoxic to the cells. There was no significant difference between 24 h and 72 h treatment with these selenocompounds. In addition, pretreatment with SS or SM for 24 or 72 h provided significant protection against the cytotoxicity caused by 3,5-DMAP and only slight differences were observed between 24 h and 72 h treatments.

As shown in Fig. 4, pretreatment with SS and SM did not cause ROS or intracellular ROS generation, compared to the control while 3,5-DMAP (25 μM) treatment caused a significant increase in intracellular ROS generation. SS and SM provided significant protection against the 3,5-DMAP-induced cytotoxicity. SS application for 24 h with 3,5-DMAP provided a decrease of 43% while SM for 24 h with

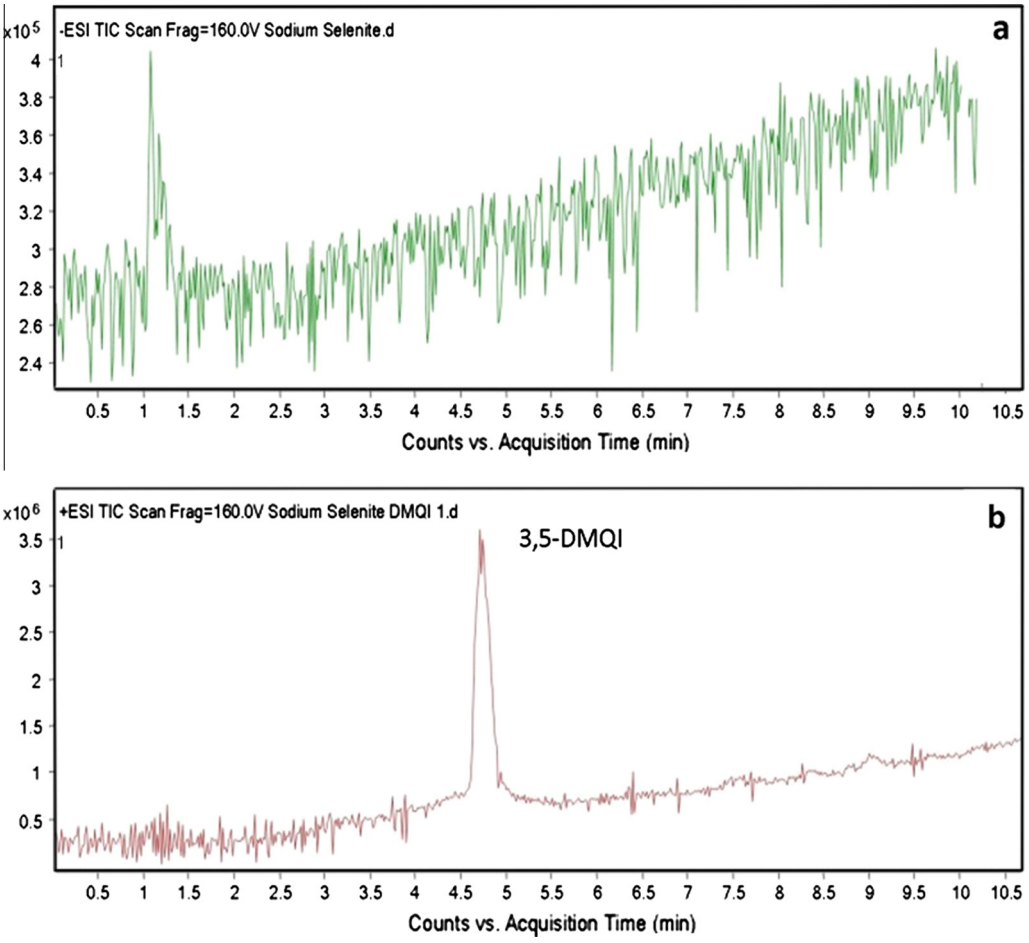

Fig. 1. Total ion chromatograms of SS (a) and 1 h incubation of SS and 3,5-DMQI (b). 3,5-DMIQ; 4-amino-3,5-dimethylphenol iminequinone; SS, sodium selenite.

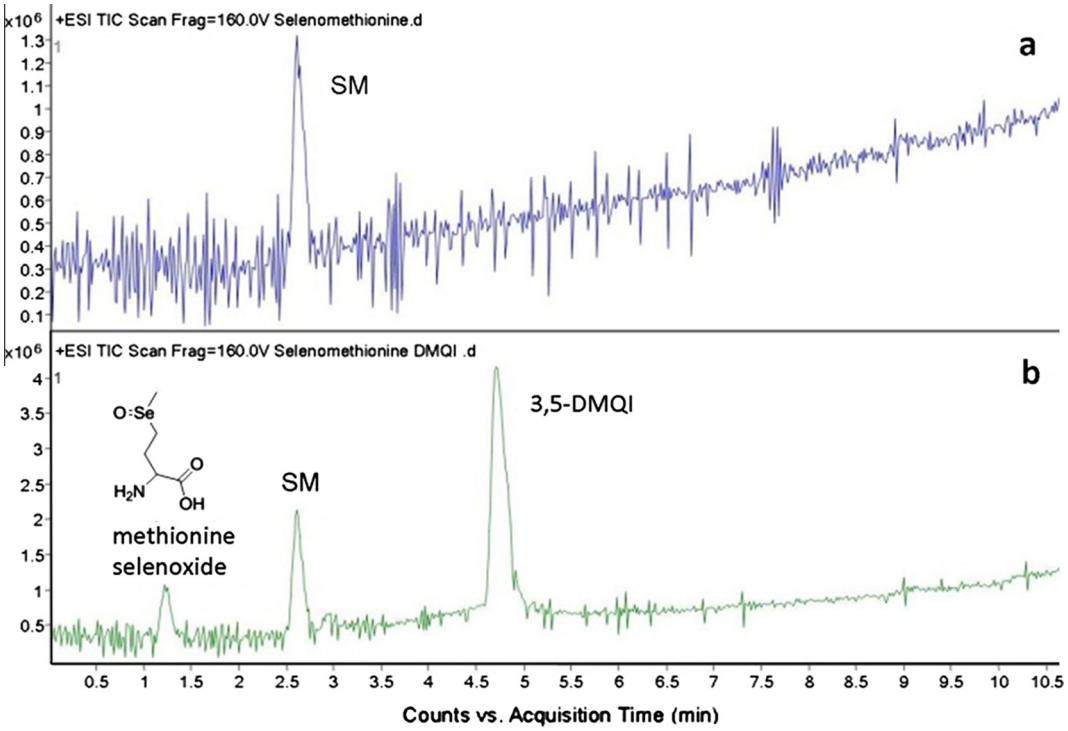

Fig. 3. Total ion chromatograms of SM (a) and 1 h incubation of SM and 3,5-3,5-DMQI (b). 3,5-DMIQ; 4-amino-3,5-dimethylphenol iminequinone; SM, selenomethionine.

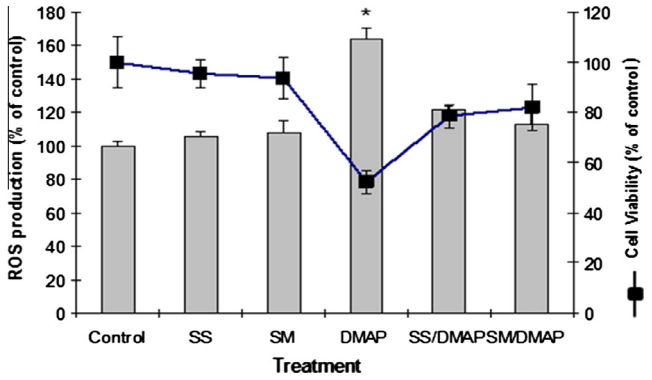

Fig. 4. Cell viability and intracellular ROS quantification in the study groups. 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite. (Control) is Control cells; (SS): AS52 cells pre-treated with SS (30 nM) for 24 h; (SM): AS52 cells pre-treated with SM (10 μM) for 24 h; (DMAP): AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SS/DMAP): AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SM/DMAP): AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. ROS, reactive oxygen species. Cytotoxicity was determined using WST-1 assay. Both cell viability and ROS production in the study groups were expressed in% of control. Bar graphs show the ROS production at 25 μM of 3,5-DMAP application while line graph shows the cell viability at 25 μM of 3,5-DMAP in study groups. Values are given as mean ± SD of $n = 3$ experiments. $p < 0.05$ was considered statistically significant. *indicates that the mean is significantly different from Control group.

3,5-DMAP supplied a decrease of 52% in cytotoxicity compared to 3,5-DMAP treatment.

3.5. Nuclear staining of 3,5-dimethylaminophenol

As shown in Fig. 5, the images A and B show the Brightfield microscopy and Hoechst 33528 nuclear stain, respectively. The arrows in Fig. 5C indicate the fluorescent spots, the locations where 3,5-DMAP was localized and generated the free radicals in the nuclei.

3.6. Oxidant and antioxidant parameters

The results of 24 h groups are shown in tables and figures since the results obtained from 24 h and 72 h supplementation groups were similar. However, in Section 3, we also mentioned the results obtained from 72 h groups where the differences between 24 h groups and 72 h groups were significant.

3.7. Activities of selenoenzymes

Table 1 shows the cytoplasmic and nuclear selenoenzyme activities in the study groups. Both SS (52%) and SM (56%) provided significant increases in cytoplasmic GPx1 activity after 24 h treatment compared to the control group. The 72 h supplementation with both of the selenocompounds supplied an increase of 75% in cytoplasmic GPx1 activity vs. control ($p < 0.05$). The differences in cytoplasmic GPx1 activity between the 24 h and 72 h treatments with both SS and SM were statistically significant. On the other hand, 3–5-DMAP treatment did not cause any change in cytoplasmic GPx1 activity, while both of the selenocompounds plus 3,5-DMAP provided marked increases in GPx1 activity vs. 3,5-DMAP group in both of the supplementation periods. The 72 h supplementations with 3,5-DMAP provided significantly higher GPx1 activities compared to 24 h groups (data not shown).

Both SS (42%) and SM (26%) supplied remarkable increases in nuclear GPx1 activity after 24 h treatment compared to control. 72 h treatments with both of the selenocompounds also supplied significant increases in nuclear GPx1 activity vs. 3,5-DMAP (SS at 62%, SM at 49%, $p < 0.05$, data not shown). The differences in nuclear GPx1 activity between the 24 h and 72 h treatments with both SS and SM were statistically significant. 3,5-DMAP treatment caused a marked decrease of 24% in nuclear GPx1 activity vs. control. Both of the selenocompounds with 3,5-DMAP treatment after

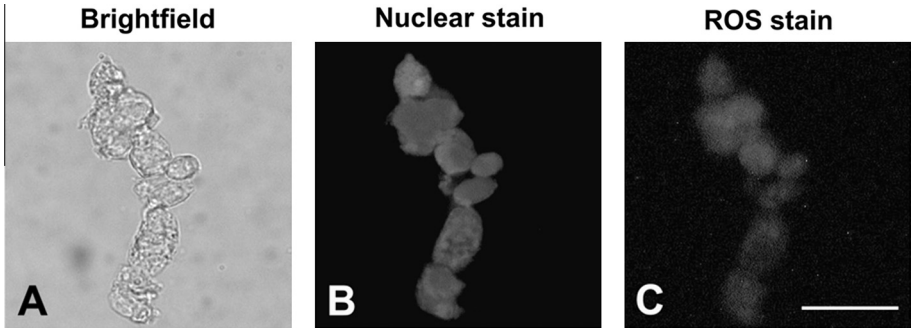

Fig. 5. Microscopy of ROS detection in nucleus. CM-H_2DCFA, 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluoresce in diacetate. ROS, reactive oxygen species. 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite. (A) Brightfield microscopy, (B) Hoechst 33528 nuclear stain, (C) indicates the fluorescent spots, the locations where 3,5-DMAP was localized and generated the free radicals in the nucleus. CM-H_2DCFDA ROS detection kit was used for ROS detection. Hoechst 33528 was used for nuclear staining. Treatment: 1 h 3,5-DMAP treatment, then 24 h fresh medium incubation. Magnification is ×400.

Table 1
Cytoplasmic and nuclear selenoenzyme activities in samples pretreated 24 h with SS or SM, followed by 1 h treatment with DMAP ± Se compounds.

	Cytoplasmic		Nuclear	
	GPx1 (U/mg protein)	TrxR (mU/mg protein)	GPx1 (U/mg protein)	TrxR (mU/mg protein)
Control	0.124 ± 0.003	0.283 ± 0.027	0.038 ± 0.001	0.028 ± 0.002
SS	0.189 ± 0.008*	0.438 ± 0.072*	0.054 ± 0.004*	0.049 ± 0.010*
SM	0.194 ± 0.006*	0.563 ± 0.053*	0.048 ± 0.002*	0.048 ± 0.007*
3,5-DMAP	0.121 ± 0.014	0.176 ± 0.024*	0.027 ± 0.001*	0.020 ± 0.005*
SS/3,5-DMAP	0.191 ± 0.030*	0.424 ± 0.062*	0.046 ± 0.002*	0.033 ± 0.010
SM/3,5-DMAP	0.213 ± 0.010*	0.383 ± 0.089*	0.041 ± 0.003	0.037 ± 0.005*

3,5-DMAP, 3,5-dimethylaminophenol; SS, sodium selenite; SM, selenomethionine.
Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements.
$p < 0.05$ was considered statistically significant.
* Indicates that the mean is significantly different from Control group.

both 24 h and 72 h treatments provided marked increases in nuclear GPx1 activity. There was an increase of in nuclear GPx1 activity (70%) in SS/DMAP group and an increase of 52% in SM/DMAP compared to 3,5-DMAP group ($p < 0.05$, both). However, no marked difference was determined between the SS/DMAP and SM/DMAP groups in the activity of nuclear GPx1 at any supplementation periods.

Both SS (55%) and SM (99%) supplied marked increases in cytoplasmic TrxR activity after 24 h treatment and 72 h treatment compared to their controls. 3,5-DMAP treatment caused a marked decrease in cytoplasmic TrxR activity (38%) vs. control. Both of the selenocompounds with 3,5-DMAP treatment (at both 24 h and 72 h) showed marked increases in TrxR activity. At 24 h, SS treatment with 3,5-DMAP provided an increase of 141% in cytoplasmic TrxR activity while SM supplementation showed an 118% increase vs. control group.

Both SS (75%) and SM (71%) supplied significant increases in nuclear TrxR activity after 24 h treatments compared to the control group. Besides, 72 h treatments with both of the selenocompounds provided further increase in nuclear TrxR activity vs. 24 h treatments. 3,5-DMAP treatment caused a marked decrease of 28% in nuclear TrxR activity compared to control. Both of the selenocompounds with 3,5-DMAP treatment provided increases in TrxR activity compared to DMAP treatment after both 24 h and 72 h supplementations. After 24 h treatment with SS or SM plus 3,5-DMAP, there was an increase of 65% in nuclear TrxR activity in SS/DMAP group and there was an increase of 85% in nuclear TrxR activity in SM/DMAP group compared to DMAP group ($p < 0.05$, both).

3.8. The activities of other antioxidant enzymes

Table 2 shows the cytoplasmic and nuclear antioxidant enzyme activities in the study groups. Supplementation with both SS and SM at 24 h and 72 h provided significant increases in cytoplasmic CAT activity compared to their controls. SS and SM supplementation for 24 h provided 49% and 30% increases in CAT activity respectively, compared to control group ($p < 0.05$, both). 3,5-DMAP treatment caused a marked decrease of 27% vs. control. Both SS (42%) and SM (39%) supplementations with 3,5-DMAP treatment provided significant increases in cytoplasmic CAT activity (at both of the supplementation periods).

Nuclear CAT activities showed marked increases by selenium supplementation. Other than 24 h SS supplementation, all of the increments were statistically significant. After 24 h, SS provided an increase of 15% while at 72 h the increase was 35% ($p < 0.05$ vs. control, both). After 24 h, SM provided an increase of 23% while after 72 h the increase in CAT activity was 28% vs. control ($p < 0.05$, both). 3,5-DMAP treatment caused a decrease of 41% in nuclear CAT activity vs. control group. Both of the selenocompounds with 3,5-DMAP treatment supplied increases in nuclear CAT activity at both of the supplementation periods compared to 3,5-DMAP treatment. There was an increase of 142% in nuclear CAT activity in SS/DMAP group while in SM/DMAP group a marked increase of 127% was observed when compared to DMAP group.

Cytoplasmic SOD activity was markedly decreased by both SS (25%) and SM (33%) supplementations after 24 h treatment. On the other hand, 3,5-DMAP treatment caused an increase of 36% in SOD activity compared to control ($p < 0.05$). Both SS (44%) and SM (41%) supplementations with 3,5-DMAP treatment caused decreases in cytoplasmic SOD activity vs. DMAP group ($p < 0.05$, both). Nuclear SOD activity was not affected by both SS and SM supplementations after 24 h ($p > 0.05$ vs. control, both). However, 3,5-DMAP treatment caused a marked increase of ~100% in nuclear SOD activity compared to control. Both SS (47%) and SM (46%) supplementations with 3,5-DMAP treatment decreased nuclear SOD activity vs. DMAP group ($p < 0.05$, both).

Cytoplasmic GR activity did not change by SS or SM in any of the supplementation periods. However, 3,5-DMAP treatment caused a

Table 2
Cytoplasmic and nuclear antioxidant enzyme activities in samples pretreated 24 h with SS or SM, followed by 1 h treatment with DMAP ±Se compounds.

	Cytoplasmic				Nuclear			
	CAT (U/ mg protein)	SOD (U/ mg protein)	GR (mU/ mg protein)	GST (nmol/ mg protein/min)	CAT (U/ mg protein)	SOD (U/ mg protein)	GR (mU/ mg protein)	GST (nmol/ mg protein/min)
Control	0.265 ± 0.019	20.965 ± 2.338	0.091 ± 0.010	27.273 ± 1.266	0.088 ± 0.009	8.967 ± 2.145	0.039 ± 0.004	3.767 ± 0.048
SS	0.395 ± 0.024*	15.776 ± 2.359*	0.111 ± 0.014	28.367 ± 0.391	0.102 ± 0.010	8.160 ± 0.584	0.040 ± 0.007	3.633 ± 0.255
SM	0.381 ± 0.008*	15.782 ± 3.025*	0.118 ± 0.020	26.614 ± 0.749	0.114 ± 0.003*	8.152 ± 1.821	0.039 ± 0.008	3.869 ± 0.231
3,5-DMAP	0.194 ± 0.019*	28.482 ± 2.729*	0.039 ± 0.014*	20.481 ± 2.024	0.052 ± 0.005*	18.984 ± 1.995*	0.019 ± 0.005*	3.345 ± 0.687
SS/3,5-DMAP	0.275 ± 0.035	15.972 ± 1.358*	0.109 ± 0.010	21.311 ± 0.727	0.126 ± 0.001*	9.980 ± 1.692	0.040 ± 0.002	3.538 ± 0.155
SM/3,5-DMAP	0.269 ± 0.047	16.927 ± 1.862*	0.114 ± 0.010	20.921 ± 1.799	0.118 ± 0.003*	10.210 ± 1.891	0.040 ± 0.005	3.758 ± 0.180

CAT, catalase; SOD, total superoxide dismutase; GR, glutathione reductase; GST, glutathione S-transferase.
3,5-DMAP, 3,5-dimethylaminophenol; SS, sodium selenite; SM, selenomethionine.
Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements. *$p < 0.05$ was considered statistically significant.
* Indicates that the mean is significantly different from Control group.

decrease of 57% when compared to DMAP group ($p < 0.05$). Both SS (180%) and SM (192%) with 3,5-DMAP treatment in both of the supplemetation periods provided increases in cytoplasmic GR activity. Nuclear GR activity did not also change by SS or SM supplementation in any of the treatment periods vs. control. However, 3,5-DMAP treatment caused a decrease of 37% ($p < 0.05$) vs. control. Both SS and SM after 24 h of supplemetation with 3,5-DMAP treatment provided 110% increase in nuclear GR activity vs. DMAP group.

We did not observe any significant GST changes in any group, either in cytoplasmic nor nuclear fractions compared to their controls.

3.9. Total, oxidized and reduced glutathione levels

Fig. 6 shows the cytoplasmic and nuclear GSH and GSSG levels of the study groups. Cytoplasmic total glutathione levels did not change significantly in any of the groups. Both SS and SM supplementations (after both 24 h and 72 h) provided marked decreases in cytoplasmic GSSG levels. 3,5-DMAP treatment caused 300% increase in GSSG levels vs. control. Both SS and SM with 3,5-DMAP treatment at both of the supplementation periods supplied decreases in GSSG levels in cytoplasm compared to 3,5-DMAP group.

3,5-DMAP caused a significant increase of 456% in nuclear GSSG levels compared to control ($p < 0.05$). SS and SM supplementations with 3,5-DMAP treatment at both of the supplementation periods provided significant decreases compared to 3,5-DMAP. After both of the supplementation periods, the decrease in GSSG levels in SS/DMAP group was ~30% and in SM/DMAP group was ~25% compared to DMAP group.

SS supplementation for 24 h provided a significant increase of 26% in GSH levels while 3,5-DMAP treatment caused a significant decrease of 35% in GSH levels when compared to the control. Along with 3,5-DMAP treatment, 24 h SS supplementation supplied an increase of 65% while 24 h SM supplementation provided significant increment of 49% when compared to DMAP group.

3.10. Thiol groups, lipid peroxidation, and protein oxidation

Fig. 7 shows the levels of TBARS (A), carbonyl groups (B) and thiol groups (C) in the study groups.

SS (42%) and SM (40%) supplementation provided significant decreases in cytoplasmic TBARS levels after 24 h compared to their control group. 3,5-DMAP caused an average of 175% increase in cytoplasmic TBARS levels vs. control while both SS (75%) and SM (70%) supplementations with 3,5-DMAP provided significant decreases when compared to DMAP group.

Only 72 h supplementations with SS (25%) and SM (17%) were effective in decreasing nuclear TBARS levels significantly (data not shown). 3,5-DMAP treatment caused an average of 713% elevation in nuclear TBARS levels compared to control group while both SS and SM supplementations with 3,5-DMAP treatment at both of the supplementation periods provided significant decreases in LP compared to DMAP group. The decrease in nuclear TBARS levels in SS/DMAP group was 83% while the decrease in SM/DMAP group was 82% vs. DMAP group ($p < 0.05$, both). However, no difference was found between 24 h and 72 h SS and SM treatments against the cytoplasmic and nuclear LP in 3,5-DMAP-treated groups.

3,5-DMAP treatment caused an elevation of 67% in cytoplasmic protein oxidation compared to control group. Both SS and SM supplementations with 3,5-DMAP application at both of the periods (24 h and 72 h) provided decreases in protein carbonyls compared to DMAP treatment. The decrease in SS/DMAP group was 56% while the decrease in SM/DMAP group was 39% (vs. DMAP group, $p < 0.05$, both).

Both SS (27%) and SM (26%) supplementations for 24 h were effective in decreasing nuclear carbonyl group levels when compared to control group ($p < 0.05$). Besides, 72 h supplementations with both of the selenocompounds were also effective in reducing nuclear protein oxidation ($p < 0.05$, vs. control). 3,5-DMAP treatment caused an average of 62% increment in nuclear carbonyl group levels compared to control. Both SS (33%) and SM (28%) supplementations for 24 h with 3,5-DMAP provided significant decreases in nuclear protein oxidation when compared to DMAP group.

SS treatment provided marked increases in both cytoplasmic and nuclear thiol levels at both of the supplementation periods vs. control. However, SM supplementation only supplied a marked increment in cytoplasmic thiol levels after 72 h of supplementation (7% vs. control, $p < 0.05$, data not shown). Other than these alterations, there were no changes in any of the groups in neither cytoplasm nor nucleus.

3.11. Caspase 3 and caspase 8 activities

Fig. 8A and B shows cellular caspase 3 and caspase 8 activities in the study groups. 3,5-DMAP treatment caused a marked increase of 34% in caspase 8 compared to control group while 24 h supplementation with both SS and SM along with 3,5-DMAP treatment provided decreases in the activity of caspase 8 vs. DMAP group. Moreover, 3,5-DMAP treatment caused a marked increase of 104% in caspase 3 activity when compared to control. SS and SM treatments with 3,5-DMAP treatment showed significant decreases in the activity of caspase 3 vs. DMAP group. We observed almost the same results with 72 h SS or SM supplementations.

3.12. Alkaline Comet assay

Fig. 9 shows olive tail moment (OTM) results in the study groups obtained from alkaline Comet assay. Both SS and SM did not provide any decrease in OTM after both 24 h and 72 h supplementations. However, 3,5-DMAP caused an increase of 299% in OTM ($p < 0.05$ vs. control). SS (49%) and SM (27%) supplementations with 3,5-DMAP treatment supplied marked decreases in OTM after 24 h supplementation (vs. DMAP group, $p < 0.05$, both). However, the differences between 24 h and 72 h of both SS and SM with 3,5-DMAP were not statistically significant.

4. Discussion

Worldwide, bladder cancer is one of the most common types of cancer (Wu et al., 2010). The established risk factors are smoking, occupational exposure to aromatic amines/alkylamines/alkylanilines, and genetics (Silverman et al., 2006; Skipper et al., 2010). Though, clearly not as carcinogenic as multi-ring aromatic amines, alkylanilines are a class of chemicals that may hold far more significance for human health than has yet been elucidated and they do not have a well-clarified toxicity mechanism (Skipper et al., 2010).

Research has been moving towards utilizing different antioxidants for preventing the genotoxic and carcinogenic effects of different environmental chemicals. Trace elements like Se are of particular interest as it is the key component of antioxidant enzymes like GPxs, which have essential roles in the cellular antioxidant defense. In the physiological dosage range, Se appears to function as an anti-mutagenic agent, preventing the malignant transformation of normal cells and the activation of oncogenes (Schrauzer, 2000). Se supplementation is protective against a wide variety of environmental chemicals and several cancers, possibly due to its effect on cellular redox equilibrium (Erkekoglu et al., 2010a, 2010b; Bera et al., 2013). In the present study, we propose

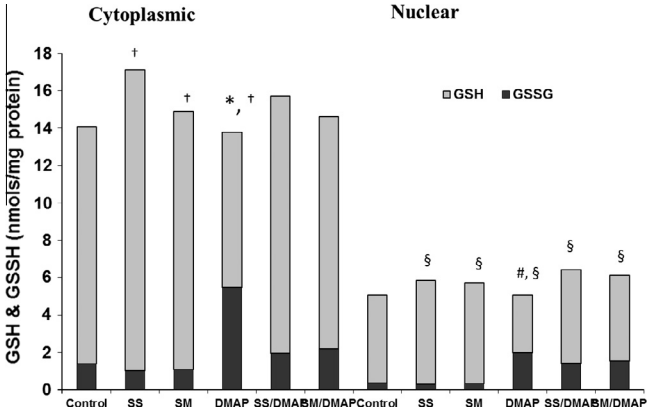

Fig. 6. Oxidized glutathione and reduced glutathione levels in the study groups. GSSG: oxidized glutathione; GSH: reduced glutathione, 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite, (Control): Control cells; (SS): AS52 cells pre-treated with SS (30 nM) for 24 h; (SM): AS52 cells pre-treated with SM (10 μM) for 24 h; (DMAP): AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SS/DMAP): AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SM/DMAP): AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. Total, oxidized and reduced glutathione levels are expressed in nmole/mg protein. Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements. * Indicates that the mean cytoplasmic GSH levels is significantly different from Control group. † Indicates that the mean cytoplasmic GSSG is significantly different from Control group. # Indicates that the mean nuclear GSH levels is significantly different from Control group. § Indicates that the mean nuclear GSSG levels is significantly different from Control group. $p < 0.05$ was considered statistically significant.

a mechanism through which alkylanilines exert their toxicity in CHO cells. In addition, we suggest that both organic and inorganic selenocompounds are protective against their toxicity. The results of this study can be discussed in three parts:

4.1. Effects of 3,5-dimethylaminophenol on the measured parameters

Excessive production of ROS may lead to cell function loss and cell death (Imai and Nakagawa, 2003). 3,5-DMAP is the major product of N-hydroxylation of 3,5-DMA. 3,5-DMAP was shown to cause DNA damage, mutagenesis and carcinogenesis (Ye et al., 2012; Chao et al., 2014). The increased intracellular ROS production with 3,5-DMAP exposure in the current study, along with substantial changes in cellular antioxidant/oxidant parameters, is the predominant evidence of a shift in the redox equilibrium towards oxidation, thus occurrence of oxidative stress. We determined that 3,5-DMAP localizes and generates free radicals in the nuclei and therefore we interrogated whether it would also cause an imbalance of antioxidant/oxidant status in nucleus among changes in the cytoplasm (Ye et al., 2012). Now, by measuring the activities of antioxidants/oxidants in both fractions, we have observed that 3,5-DMAP exerts its toxicity on both fractions, where the nuclear toxicity seems to be more pronounced.

In CHO cells, 3,5-DMAP toxicity was evidenced by changes in both cytoplasmic and nuclear selenoenymes. Nuclear GPx1 activity, as well as cytoplasmic and nuclear TrxR activities markedly decreased vs. control whereas cytoplasmic GPx1 activity was not affected. It was shown that GPx1 was induced in order to overcome the oxidative burden in different conditions (Kim et al., 2011; Erkekoglu et al., 2014); however this is not the case in the present study. Although 3,5-DMAP does not affect cytosolic GPx1 activity, it causes induction of H_2O_2 (Chao et al., 2012, 2014). H_2O_2 can produce hydroxyl radical which is the most dangerous type of ROS. Therefore, it is clear that oxidative stress might arise due to high ROS production and the unchanged GPx1 actvity after 3,5-DMAP exposure. On the other hand, 3,5-DMAP caused significant decreases in both cytoplasmic and nuclear CAT activity, while causing substantial elevations in SOD activity in the two fractions. We can postulate that the superoxide production might be induced by 3,5-DMAP, triggering the mechanism of superoxide dismutation by SOD. The increased activity of SOD might be related to this assumption, at least in part. However, other oxygen species like H_2O_2 and. OH are also be induced by 3,5-DMAP as shown previously (Chao et al., 2014). As both nuclear CAT and GPx1 activity decreases, the decomposition of H_2O_2 could not be achieved and the increases in cellular ROS levels might have both cytotoxic and genotoxic effects on CHO cells. GSH, the most important intracellular thiol, is a substrate for the GPxs, and GSTs (Dolphin et al., 1989). GR provides adequate levels of cellular GSH by reducing GSSG (Dolphin et al., 1989). The increase was observed in GSSG levels, and the significant decrease in GSH concentrations and cellular redox ratio with 3,5-DMAP treatment might be related to the decrease in GR activity.

The increased intracellular levels of LP is a good indicator of intracellular oxidation (Alía et al., 2006; Mateos and Bravo, 2007). During LP, unstable hydroperoxides, resulting from peroxyl radical-dependent chain reactions among unsaturated fatty acyl moieties break down to smaller and more stable products [e.g. aldehydes, such as acrolein, malonyldialdehyde (MDA) and 4-hydroxynonenal (HNE)] (Emerit et al., 1991). These products can attack several cellular targets, like proteins. Carbonyl groups may be introduced into proteins by reactions with MDA or HNE. Besides, direct oxidation of lysine, arginine, proline, and threonine residues may yield carbonyl derivatives (Esterbauer et al., 1991; Uchida et al., 1993; Winkler et al., 1984). Moreover, MDA and HNE can interact with DNA, thereby inducing mutagenecity and

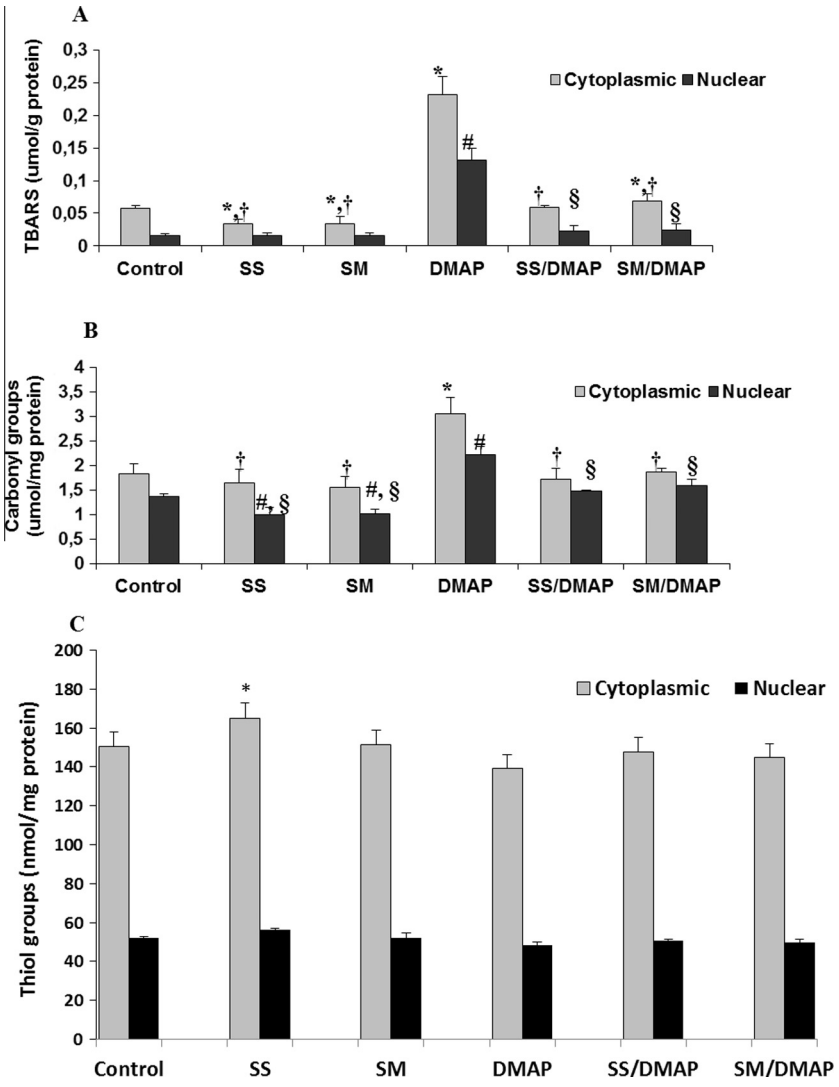

Fig. 7. Thiol groups, lipid peroxidation and protein oxidation levels in the study groups. (A) TBARS levels. (B) Carbonyl levels. (C) Thiol groups. TBARS: thiobarbituric acid reactive substance. 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite, (Control): Control cells; (SS): AS52 cells pre-treated with SS (30 nM) for 24 h; (SM): AS52 cells pre-treated with SM (10 μM) for 24 h; (DMAP): AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SS/DMAP): AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SM/DMAP): AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements. $p < 0.05$ was considered statistically significant. * Indicates that the mean cytoplasmic TBARS or carbonyl group level is significantly different from Control group. † Indicates that the mean cytoplasmic TBARS or carbonyl group level is significantly different from DMAP group. # Indicates that the mean nuclear TBARS or carbonyl group level is significantly different from control group. § Indicates that the mean nuclear TBARS or carbonyl group level is significantly different from DMAP group. TBARS levels are expressed in μmole/g protein and carbonyl group levels were expressed in μmole/mg protein.

carcinogenicity (de Zwart et al., 1999). Currently, both LP and protein oxidation showed significant increases by 3,5-DMAP treatment and we might suggest that LP might be one of the underlying factors for the increase in protein oxidation after 3,5-DMAP treatment in AS52 cells (Reinheckel et al., 1998).

For genotoxicity determination, a high through-put "Comet Chip" protocol is used with a newly developed automated image analysis software. A limitation of the traditional assay is that each sample requires a separate glass slide and image analysis is laborious and data is intensive, thus reducing throughput. This new technique uses micro-fabrication technologies to enable analysis of cells within a defined array, resulting in a >200 fold reduction in the area required per condition (Weingeist et al., 2013). With this new technique, we had the opportunity to extensively study the genotoxicity of 3,5-DMAP in AS52 and UV5 CHO cells (Chao et al., 2012). We observed that both %tail DNA (data not shown) and OTM increased significantly by 3,5-DMAP treatment, indicating this particular alkylaniline induces genotoxicity in CHO cells. To our knowledge, little is known regarding the mechanism of genotoxicity of 3,5-DMA or 3,5-DMAP. However, there is evidence that 3,5-DMA forms DNA adducts and has a greater carcinogenic potential than two other significant alkylanilines, namely 2,6-DMA, and 3-EA (Skipper et al., 2006).

The concerted action of caspases is responsible for apoptosis. There are two main caspase-activation cascades described for apoptosis: the intrinsic and extrinsic apoptotic pathways (Dang, 2012). When activated, the extrinsic pathway, which includes the action of initiator caspases, 8 and 10, cleave and activate the executioner caspases 3 and 7 (Boatright and Salvesen, 2003). It was demonstrated that oxidative stress can markedly activate caspase 8. Herein, we observe marked increases in both caspase 8 and 3 activities (Baumgartner et al., 2007), suggesting extrinsic caspase pathway is induced by 3,5-DMAP. Therefore, we might postulate that 3,5-DMAP triggers apoptosis in CHO AS52 cells, by inducing

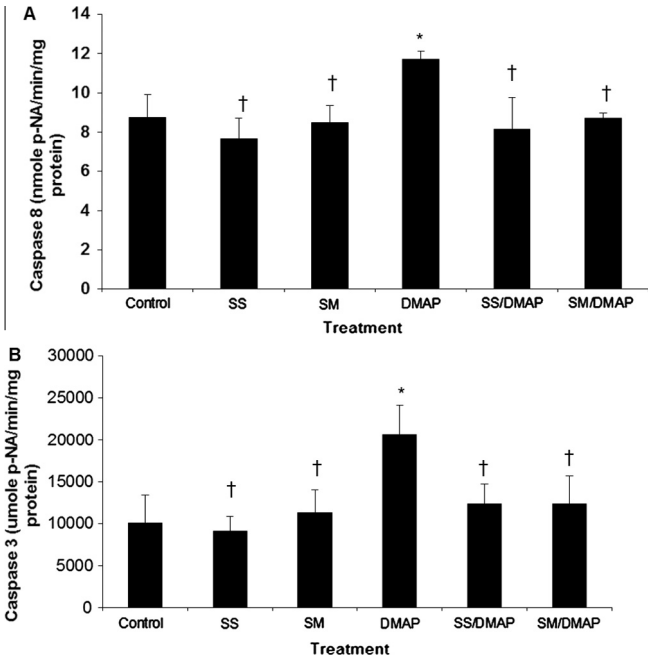

Fig. 8. Caspase 3 and Caspase 8 activity. 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite, (Control): Control cells; (SS): AS52 cells pre-treated with SS (30 nM) for 24 h; (SM): AS52 cells pre-treated with SM (10 μM) for 24 h; (DMAP): AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SS/DMAP): AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SM/DMAP): AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. Caspase 8 activity is expressed as nmole/p-NA/min/mg protein. Caspase 3 activity is expressed as μmole/p-NA/min/mg protein. Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements. $p < 0.05$ was considered statistically significant. * Indicates that the mean is significantly different from C 24 group. † Indicates that the mean is significantly different from DMAP 24 group.

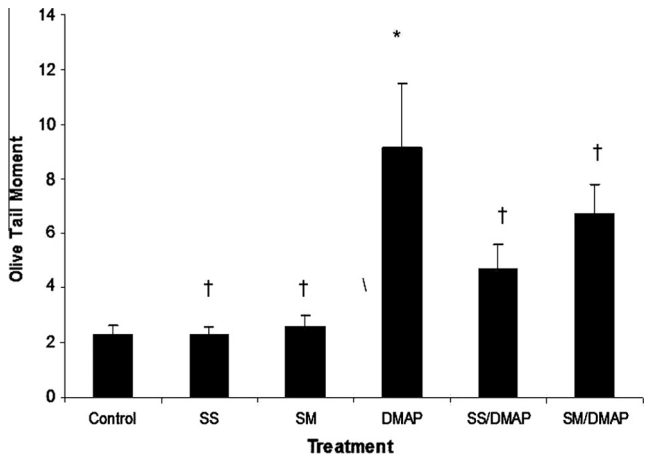

Fig. 9. OTM values obtained from alkaline Comet assay. OTM, Olive Tail Moment, 3,5-DMAP, 3,5-dimethylaminophenol; SM, selenomethionine; SS, sodium selenite, (Control) is Control cells; (SS): AS52 cells pre-treated with SS (30 nM) for 24 h; (SM): AS52 cells pre-treated with SM (10 μM) for 24 h; (DMAP): AS52 cells for 24 h groups; cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SS/DMAP): AS52 cells were pre-treated with SS (30 nM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h; (SM/DMAP): AS52 cells pre-treated with SM (10 μM) for 24 h; later cells were treated with 25 μM of 3,5-DMAP (in serum free medium) for 1 h. Values are given as mean ± SD of $n = 3$ experiments and duplicate measurements. $p < 0.05$ was considered statistically significant. * Indicates that the mean is significantly different from C 24 group. † Indicates that the mean is significantly different from DMAP 24 group.

caspase 8 activity, which in turn induces caspase 3. We have also shown that 3,5-DMAP induces pro-caspase 3 and pro-PARP expressions before (Chao et al., 2014). We can suggest that oxidative stress induced by 3,5-DMAP can be one of the underlying mechanisms of genotoxicity as well as apoptotic cell death herein.

4.2. Effects of selenocompounds on measured parameters

Although most of its chemopreventive mechanisms of Se still remain unclear, the protective effects of Se seem to be primarily associated with its presence in GPxs, which are known to protect DNA and other cellular components from damage by oxygen radicals (Negro, 2008). Moreover, mammalian TrxR enzymes are also important antioxidant selenoenzymes, that contain Se-containing pyridine nucleotide-disulfide oxidoreductases with mechanistic and sequence identity, including a conserved -Cys-Val-Asn-Val-Gly-Cys- redox catalytic site, to GRs. TrxRs catalyze the NADPH-dependent reduction of the redox protein thioredoxin (Trx) as well as of a wide number of exogenous and endogenous compounds. The importance of Trx to many aspects of cell function make it likely that TrxRs also play a role in protection against oxidant injury, cell growth and transformation, and the recycling of ascorbate from its oxidized form (Mustacich and Powis, 2000). Therefore, Se plays several roles in carcinogen metabolism (Schrauzer, 2000). Low dietary Se intake makes the organism prone to oxidative stress-related conditions, reduced fertility and immune functions (Ganther, 1999). Moreover, epidemiological studies have shown an inverse relationship between Se intake and cancer incidence, including bladder cancer (Rayman, 2005; Wallace et al., 2009). It appears that Se has multi-layered effects on DNA integrity involving anti-oxidative defenses, on oxidative stress, and DNA repair proteins (Hartwig et al., 2003). However, 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, mutagenic and possibly carcinogenic (Letavayová et al., 2006).

The protection efficiency of Se derivatives strongly depends on the chemical form. SS is commonly used in cell culture and animal studies, and SM is the most common in the diet. SM is converted to 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. 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 (Zhuo et al., 2009), thus providing lower dose supplementation with SS (30 nM), compared to SM (10 μM), with almost the same increment in the selenoenzyme activities. Supplementation of cells with physiologically relevant concentrations of SS and SM was shown to protects against the cytotoxicity and genotoxicity; however protection against other types of stress (i.e. alkylating or radiating agents) could not be achieved by these selenocompounds (Erkekoglu et al., 2010a, 2010b; de Rosa et al., 2012).

The concentrations of SS and SM used in this study were chosen from preliminary experiments, considering maximum GPx1 induction after 24 h and 72 h. These concentrations were also in the same ranges that were applied previously for several other cell types (Chu et al., 1990; Bhamre et al., 2003; Mansur et al., 2000). Both SS and SM supplied substantial increases in nuclear GPx1 and TrxR activities in both of the supplementation periods, where the increases were much more prononced in 72 h. Se supplementation was also shown to increase GPx1 and TrxR activities in other cell types after 72 h (Erkekoglu et al., 2010a, 2010b).

After treatment with both of the selenocompounds, we observed that cytoplasmic SOD activity showed significant decreases and cytoplasmic CAT activity showed marked increases

compared to control cells. However, both nuclear SOD and nuclear CAT activities did not show significant alterations after application of SS or SM. Overall results suggest that both of the selenocompounds provided increases in cellular redox ratio at both 24 and 72 h, providing higher GSH and lower GSSG levels, as well as higher thiol groups. This might be according to increased GR activity (although not significantly different vs. control). Additionally, LP was significantly reduced by both SS and SM at both of the application periods in cytoplasm while in nucleus only after 72 h, a marked decrease in LP was achieved by both SS and SM supplementations. However, in nucleus both SS and SM provided decreases in protein oxidation at both of the supplementation periods. No appreciable changes were observed in genotoxicity and caspase activities with SS and SM. These results suggest that both SS and SM were not toxic at the doses used and provided significant recruitment in both cytoplasmic and nuclear antioxidant/oxidant balance.

4.3. Effects of selenocompounds on 3,5-dimethylaminophenol toxicity

In both fractions, both SS and SM supplied increases in both GPx1 and TrxR activities, compared to 3,5-DMAP, at 24 h and 72 h. Although 3,5-DMAP does not cause any change in cytoplasmic GPx1 activity, it still causes production of H_2O_2. When we apply SS or SM with 3,5-DMAP, the GPx1 activity increases which gives the cells the capacity to overcome the oxidative stress produced by this particular alkylaniline metabolite.

Both of the selenocompounds with 3,5-DMAP also provided substantial increases in both CAT and GR in nucleus and cytoplasm, vs. 3,5-DMAP. Besides, SOD activity decreased in both SS/DMAP and SM/DMAP groups in both of the supplementation periods, possibly indicating a decrease in superoxide production in both cytoplasm and nucleus. Both of SS and SM plus 3,5-DMAP supplied significantly higher GSH/GSSG redox ratio compared to 3,5-DMAP group, by providing decreases in GSSG and increases in GSH in both of the cellular fractions. The protection seems to be more pronounced in the cytoplasm. Regarding total thiol groups, there were no changes in the cellular concentration of thiol groups in DMAP-treated cells. Although GSH is the dominant thiol within the cell, other thiols are also present (thioredoxin, peroxiredoxin, cysteine and cysteamine pools, pantethine, etc) (Akerboom and Sies, 1981). We might postulate that these thiols are not suppressed by 3,5-DMAP. It was observed that elevated levels of thioredoxin have also been observed in several human bladder and prostatic cancer cell lines (Yokomizo et al., 1995) and this might point out that in disease conditions or oxidative stress-related pathologies thiols like thioredoxin might increase and in our case this might compensate the decrease in GSH leading to no changes in total cellular thiol concentration.

Prevention of LP plays an important role in maintaning tissue integrity (Pamplona, 2008). On the other hand, LP as well as protein oxidation decreased in SS/DMAP and SM/DMAP groups when they were compared to 3,5-DMAP group. Moreover, caspase activities decreased to almost the control levels, while OTM decreased significantly in both SS/DMAP and SM/DMAP groups vs. 3,5-DMAP. SS supplementation was more protective vs. SM treatment.

In conclusion, this is the first study showing the oxidative potential of 3,5-DMAP in CHO AS52 cells, as well as providing evidence that selenocompounds can be protective against its toxicity. After 24 h of supplementation, both SS and SM provided substantial changes in antioxidant/oxidant status of the 3,5-DMAP-treated cells; however other than supplying higher GPx1 and TrxR activities, 72 h supplementation did not provide any advanced improvement. Considering the cyto- and genotoxic effects of 3,5-DMAP, it can be postulated that even mildly Se-deficient individuals might be more susceptible to the adverse effects of alkylanilines. 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 alkylaniline exposures and their toxic effects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2014.06.031.

References

Akerboom, T.P., Sies, H., 1981. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. Methods Enzymol. 77, 373–382.
Alía, M., Ramos, S., Mateos, R., et al., 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. Toxicol. Appl. Pharmacol. 212 (2), 110–111.
Amaral, A.F. et al., 2010. Selenium and bladder cancer risk: a meta-analysis. Cancer Epidemiol. Biomarkers Prev. 19 (9), 2407–2415.
Arnér, E.S., Zhong, L., Holmgren, A., 1999. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. Methods Enzymol. 300, 226–239.
Baliga, M.S., Wang, H., Zhuo, P., et al., 2007. Selenium and GPx-1 overexpression protect mammalian cells against UV-induced DNA damage. Biol. Trace Elem. Res. 115 (3), 227–242.
Baumgartner, H.K., Gerasimenko, J.V., Thorne, C., et al., 2007. Caspase-8-mediated apoptosis induced by oxidative stress is independent of the intrinsic pathway and dependent on cathepsins. Am. J. Physiol. Gastrointest. Liver Physiol. 293 (1), G296–G307.
Bera, S., De Rosa, V., Rachidi, W., et al., 2013. Does a role for selenium in DNA damage repair explain apparent controversies in its use in chemoprevention? Mutagenesis 28 (2), 127–134.
Bhamre, S., Whitin, J.C., Cohen, H.J., 2003. Selenomethionine does not affect PSA secretion independent of its effect on LNCaP cell growth. Prostate 54 (4), 315–321.
Boatright, K.M., Salvesen, G.S., 2003. Mechanisms of caspase activation. Curr. Opin. Cell Biol. 15 (6), 725–731.
Chao, M.W., Erkekoglu, P., Tseng, C.Y., et al., 2014. Intracellular generation of ROS by 3,5-dimethylaminophenol:persistence, cellular response, and impact of molecular toxicity. Toxicol. Sci.. http://dx.doi.org/10.1093/toxsci/kfu127.
Chao, M.W., Kim, M.Y., Ye, W., et al., 2012. Genotoxicity of 2,6- and 3,5-dimethylaniline in cultured mammalian cells: the role of reactive oxygen species. Toxicol. Sci. 130 (1), 48–59.
Chu, F.F., Esworthy, R.S., Akman, S., et al., 1990. Modulation of glutathione peroxidase expression by selenium: effect on human MCF-7 breast cancer cell transfectants expressing a cellular glutathione peroxidase cDNA and doxorubicin-resistant MCF-7 cells. Nucleic Acids Res. 18 (6), 1531–1539.
Dalton, T.P., Shertzer, H.G., Puga, A., 1999. Regulation of gene expression by reactive oxygen. Annu. Rev. Pharmacol. Toxicol. 39, 67–101.
Dang, T.P., 2012. Notch, apoptosis and cancer. Adv. Exp. Med. Biol. 727, 199–209.
de Rosa, V., Erkekoglu, P., Forestier, A., et al., 2012. Low doses of selenium specifically stimulate the repair of oxidative DNA damage in LNCaP prostate cancer cells. Free Radic. Res. 46 (2), 105–116.
de Zwart, L.L., Meerman, J.H., Commandeur, J.N., et al., 1999. Biomarkers of free radical damage experimental animals and in applications in humans. Free Radic. Biol. Med. 26 (1–2), 202–226.
Dolphin, D., Poulson, R., Avramovic, O. (Eds.), 1989. Glutathione: Chemical, Biochemical and Metabolic Aspects, A and B. John Wiley and Sons, New York.
Dröge, W., 2002. The plasma redox state and ageing. Ageing Res. Rev. 1 (2), 257–278.

el-Bayoumy, K., Upadhyaya, P., Date, V., et al., 1991. Metabolism of [14C]benzyl selenocyanate in the F344 rat. Chem. Res. Toxicol. 4 (5), 560–565.
Emerit, I., Khan, S.H., Esterbauer, H., 1991. Hydroxynonenal, a component of clastogenic factors? Free Radic. Biol. Med. 10 (6), 371–377.
Erkekoglu, P., Rachidi, W., De Rosa, V., et al., 2010a. Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate treatment in LNCaP cells. Free Radic. Biol. Med. 49 (4), pp. 559-556.
Erkekoglu, P., Rachidi, W., Yuzugullu, O.G., et al., 2010b. Evaluation of cytotoxicity and oxidative DNA damaging effects of di(2-ethylhexyl)-phthalate (DEHP) and mono(2-ethylhexyl)-phthalate (MEHP) on MA-10 Leydig cells and protection by selenium. Toxicol. Appl. Pharmacol. 248 (1), 52–62.
Erkekoglu, P., Giray, B., Rachidi, W., Hininger-Favier, I., Roussel, A.M., Favier, A., Hincal, F., 2014. Effects of di(2-ethylhexyl)phthalate on testicular oxidant/antioxidant status in selenium-deficient and selenium-supplemented rats. Environ. Toxicol. 29 (1), 98–107.
Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11 (1), 81–128.
Fishbein, J.C., McClelland, R.A., 1987. Azide ion trapping of the intermediate in the scheme I Bamberger Rearrangement. Lifetime of a free nitrenium ion in aqueous solution. J. Am. Chem. Soc. 109, 2824–2825.
Flohé, L., Günzler, W.A., 1984. Assays of glutathione peroxidase. Methods Enzymol. 105, 114–121.
Ganther, H.E., 1999. Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. Carcinogenesis 20 (9), 1657–1666.
Goldberg, D.M., Spooner, R.J., 1983. Glutathione reductase. In: Bergmeyer, H.U., Bergmeyer, J., Grabl, M. (Eds.), Methods of Enzymatic Analysis. Verlag Chemie, Weinheim, pp. 258–265 (third ed., vol. 111).
Günzler, W.A., Kremers, H., Flohé, L., 1974. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. Z. Klin. Chem. Klin. Biochem. 12 (10), 444–448.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249 (22), 7130–7139.
Hartwig, A., Blessing, H., Schwerdtle, T., et al., 2003. Modulation of DNA repair processes by arsenic and selenium compounds. Toxicology 193 (1–2), 161–169.
Imai, H., Nakagawa, Y., 2003. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radic. Biol. Med. 34 (2), 145–169.
Jablonska, E., Gromadzinska, J., Reszka, E., et al., 2009. Association between GPx1 Pro198Leu polymorphism, GPx1 activity and plasma selenium concentration in humans. Eur. J. Nutr. 48 (6), 383–386.
Jefferies, P.R., Quistad, G.B., Casida, J.E., 1998. Dialkylquinonimines validated as in vivo metabolites of alachlor, acetochlor, and metolachlor herbicides in rats. Chem. Res. Toxicol. 11 (4), 353–359.
Kern, J.C., Kehrer, J.P., 2005. Free radicals and apoptosis: relationships with glutathione, thioredoxin, and the BCL family of proteins. Front. Biosci. 10, 1727–1738.
Kim, R.O., Rhee, J.S., Won, E.J., Lee, K.W., Kang, C.M., Lee, Y.M., Lee, J.S., 2011. Ultraviolet B retards growth, induces oxidative stress, and modulates DNA repair-related gene and heat shock protein gene expression in the monogonont rotifer, Brachionus sp. Aquat. Toxicol. 101 (3–4), 529–539.
Krieg, R.C., Dong, Y., Schwamborn, K., et al., 2005. Protein quantification and itstolerance for different interfering reagents using the BCA-method with regard to 2D SDS PAGE. J. Biochem. Biophys. Methods 65 (1), 13–19.
Lee, S.A., 2009. Gene-diet interaction on cancer risk in epidemiological studies. J. Prev. Med. Public Health 42 (6), 360–370.
Letavayová, L., Vlcková, V., Brozmanová, J., 2006. Selenium: from cancer prevention to DNA damage. Toxicology 227 (1–2), 1–14.
Mansur, D.B., Hao, H., Gladyshev, V.N., et al., 2000. Multiple levels of regulation of selenoprotein biosynthesis revealed from the analysis of human glioma cell lines. Biochem. Pharmacol. 60 (4), 489–497.
Martindale, J.L., Holbrook, N.J., 2002. Cellular response to oxidative stress: signaling for suicide and survival. J. Cell. Physiol. 192 (1), 1–15.
Mateos, R., Bravo, L., 2007. Chromatographic and electrophoretic methods for theanalysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). J. Sep. Sci. 30 (2), 175–191.
Mustacich, D., Powis, G., 2000. Thioredoxin reductase. Biochem. J. 346 (Pt 1), 1–8.
Negro, R., 2008. Selenium and thyroid autoimmunity. Biologics 2 (2), 265–273.
Pamplona, R., 2008. Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. Biochim. Biophys. Acta 1777 (10), 1249–1262.
Rayman, M.P., 2005. Selenium in cancer prevention: a review of the evidence and mechanism of action. Proc. Nutr. Soc. 64 (4), 527–542.
Reinheckel, T., Nedelev, B., Prause, J., et al., 1998. Occurrence of oxidatively modified proteins: an early event in experimental acute pancreatitis. Free Radic. Biol. Med. 24 (3), 393–400.
Reuter, S., Gupta, S.C., Chaturvedi, M.M., et al., 2010. Oxidative stress, inflammation, and cancer: how are they linked? Free Radic. Biol. Med. 49 (11), 1603–1616.
Richard, M.J., Portal, B., Meo, J., et al., 1992. Malondialdehyde kit evaluated for determining plasma and lipoprotein fractions that react with thiobarbituric acid. Clin. Chem. 38 (5), 704–709.
Schrauzer, G.N., 2000. Anticarcinogenic effects of selenium. Cell. Mol. Life Sci. 57, 1864–1873.
Silverman, D., Devesa, S., Moore, L., et al., 2006. Bladder cancer. In: Schottenfeld, D., Fraumeni, J., Jr. (Eds.), Cancer Epidemiology and Prevention, 3rd ed. Oxford University Press, New York, pp. 1101–1127.
Sharma, S., Raghuvanshi, B.P., Shukla, S., 2014. Toxic effects of lead exposure in rats:involvement of oxidative stress, genotoxic effect, and the beneficial role of N-acetylcysteine supplemented with selenium. J. Environ. Pathol. Toxicol. Oncol. 33 (1), 19–32.
Skipper, P.L., Kim, M.Y., Sun, H.L., et al., 2010. Monocyclic aromatic amines as potential human carcinogens: old is new again. Carcinogenesis 31 (1), 50–58.
Skipper, P.L., Trudel, L.J., Kensler, T.W., et al., 2006. DNA adduct formation by 2,6-dimethyl-, 3,5-dimethyl-, and 3-ethylaniline in vivo in mice. Chem. Res. Toxicol. 19 (8), 1086–1090.
Sone, T., Hamamoto, K., Seiji, Y., et al., 1981. Kinetics and mechanisms of the Bamberger Rearrangement. Part 4. Rearrangement of sterially hindered phenylhydroxylamines to 4-aminophenols in aqueous sulphuric acid solution. J. Chem. Soc. Perkin. Trans. 2, 1596–1598.
Soory, M., 2009. Relevance of nutritional antioxidants in metabolic syndrome, ageing and cancer: potential for therapeutic targeting. Infect. Disord. Drug Targets 9 (4), 400–414.
Uchida, K., Szweda, L.I., Chae, H.Z., et al., 1993. Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. Proc. Natl. Acad. Sci. USA 90 (18), 8742–8746.
Wallace, K., Kelsey, K.T., Schned, A., et al., 2009. Selenium and risk of bladder cancer: a population-based case-control study. Cancer Prev. Res. (Phila.) 2 (1), 70–73.
Weingeist, D.M., Ge, J., Wood, D.K., et al., 2013. Single-cell microarray enables high-throughput evaluation of DNA double-strand breaks and DNA repair inhibitors. Cell Cycle 12 (6), 907–915.
Winkler, P., Lindner, W., Esterbauer, H., et al., 1984. Detection of 4-hydroxynonenal as a product of lipid peroxidation in native Ehrlich ascites tumor cells. Biochim. Biophys. Acta 796 (3), 232–237.
Wood, D.K., Weingeist, D.M., Bhatia, S.N., et al., 2010. Single cell trapping and DNA damage analysis using microwell arrays. Proc. Natl. Acad. Sci. USA 107 (22), 10008–10013.
Wu, J.T., Han, B.M., Yu, S.Q., et al., 2010. Androgen receptor is a potential therapeutic target for bladder cancer. Urology 75 (4), 820–827.
Ye, W., Seneviratne, U.I., Chao, M.W., et al., 2012. Transimination of quinone imines: a mechanism for embedding exogenous redox activity into the nucleosome. Chem. Res. Toxicol. 25 (12), 2627–2629.
Yoshida, R., Oikawa, S., Ogawa, Y., et al., 1998. Mutagenicity of p-aminophenol in *E. coli* WP2uvrA/pKM101 and its relevance to oxidative DNA damage. Mutat. Res. 415 (1–2), 139–150.
Yokomizo, A., Ono, M., Nanri, H., et al., 1995. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. Cancer Res. 55 (19), 4293–4296.
Zhuo, P., Goldberg, M., Herman, L., et al., 2009. Molecular consequences of genetic variations in the glutathione peroxidase 1 selenoenzyme. Cancer Res. 69 (20), 8183–8190.