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Antioxidants and selenocompounds inhibit 3,5-dimethylaminophenol toxicity to human urothelial cells

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Exposure to alkyl anilines may lead to bladder cancer, which is the second most frequent cancer of the urogenital tract. 3,5-dimethylaniline is highly used in industry. Studies on its primary metabolite 3,5-dimethylaminophenol (3,5-DMAP) showed that this compound causes oxidative stress, changes antioxidant enzyme activities, and leads to death of different mammalian cells. However, there is no *in vitro* study to show the direct effects of 3,5-DMAP on human bladder and urothelial cells. Selenocompounds are suggested to decrease oxidative stress caused by some chemicals, and selenium supplementation was shown to reduce the risk of bladder cancer. The main aim of this study was to investigate whether selenocompounds organic selenomethionine (SM, 10 µmol/L) or inorganic sodium selenite (SS, 30 nmol/L) could reduce oxidative stress, DNA damage, and apoptosis in UROtsa cells exposed to 3,5-DMAP. 3,5-DMAP caused a dose-dependent increase in intracellular generation of reactive oxygen species, and its dose of 50 µmol/L caused lipid peroxidation, protein oxidation, and changes in antioxidant enzyme activities in different cellular fractions. The comet assay also showed single-strand DNA breaks induced by the 3,5-DMAP dose of 50 µmol/L, but no changes in double-strand DNA breaks. Apoptosis was also triggered. Both selenocompounds provided partial protection against the cellular toxicity of 3,5-DMAP. Low selenium status along with exposure to alkyl anilines can be a major factor in the development of bladder cancer. More mechanistic studies are needed to specify the role of selenium in bladder cancer.

KEY WORDS: alkyl anilines; cytotoxicity; genotoxicity; reactive oxygen species; selenium; sodium selenite; selenomethionine

Bladder cancer is the sixth most common cancer in men and the 17th in women. Almost 550,000 new cases were reported in the world in 2018 alone (1–3). It accounts for about 7% of all cancer incidence in men. In the European Union countries, it is the fourth most common cancer among men (4). Annual incidence is 36/100,000 in men and 10/100,000 in women, and mortality 13/100,000 in men, and 4/100,000 in women. Its incidence and prevalence rise after the age of 60 (5).

Well-established risk factors are tobacco consumption, occupational exposure (particularly to monocyclic aromatic amines and polycyclic aromatic hydrocarbons), and infection with *Schistosoma hematobium* (6, 7). Other risk factors include alcohol consumption, high coffee consumption, low fruit and vegetable consumption, low vitamin and trace element intake (low selenium and vitamin E intake in particular), exposure to drinking water contaminated with environmental chemicals, and some medical treatments (7–9).

Exposure to environmental alkyl amines/alkyl anilines, aniline dyes, and other aromatic amines, which originate from multiple sources, is also a well-documented risk factor for this disease. For example, tobacco smoke, which contains high levels of aromatic amines, increases the risk of bladder cancer from two to five times (1). These chemicals are also present in hair dyes, combustion products, pharmaceuticals, and pesticides (8).

Workers exposed to o-toluidine, aniline, and nitrobenzene at a rubber chemical manufacturing plant in New York had nearly three times higher bladder cancer incidence than the New York State general population. Smoking habits were suggested to account for only 8% higher bladder cancer incidence in that cohort (7). A non-occupational epidemiological study in the Los Angeles County showed that 2,6-dimethylaniline (2,6-DMA),

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3,5-dimethylaniline (3,5-DMA), and 3-ethylaniline (3-EA), were significantly associated with bladder cancer incidence, regardless of smoking (10).

3,5-dimethylaniline is used in the production of azo dyes, pharmaceuticals, antioxidants, detergents, wood preservatives, textiles, metal complexes, and antiozonants (8). Like other aromatic amines, 3,5-DMA is metabolised by cytochrome P450 enzymes (CYP450) through N-hydroxylation. N-hydroxy-3,5-dimethylaniline (N-OH-3,5-DMA) is a direct mutagen, supporting the evidence that N-hydroxylation of such compounds is a bio-activation step. The parent compound, 3,5-DMA, has also been shown to bind to DNA in laboratory animals, but there is no sufficient evidence of DNA adduct formation in humans (11). 3,5-dimethylaminophenol (3,5-DMAP), in turn, is either a product of 3,5-DMA oxidation or a N-OH-3,5-DMA hydroxylation metabolite catalysed by CYP450. 3,5-DMAP can be metabolised into 3,5-dimethylquinone imine (3,5-DMQI), which undergoes further redox cycling, whose by-products are reactive oxygen species (ROS), H_2O_2 and hydroxyl (•OH) radicals in particular (12).

In our earlier studies (11–14), 3,5-DMAP induced ROS production, cytotoxicity, and genotoxicity in Chinese hamster ovary (CHO) cells. It also up-regulated caspase 3 and 8 activities and activated apoptosis.

If not counteracted by antioxidants and intracellular antioxidant systems, ROS damages important cellular molecules, including proteins, lipids, and DNA (15). Low antioxidant levels, low selenium in particular, increase the risk of several types of malignancies, including bladder cancer (16, 17). Selenium is a component of several antioxidant enzymes, namely glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) (18, 19). We have previously shown that organic and inorganic selenium protect against oxidative stress produced by a wide variety of chemicals (20, 21).

Recently, we found that *N*-acetylcysteine (NAC), ascorbic acid (Asc), and selenocompounds protect modified CHO cells, including gpt-transgenic As52, aprt-transgenic AA8, and aprt-transgenic/NER-deficient UV5 cells, from oxidative damage caused by 3,5-DMAP (12–14). However, no effects of alkyl amine/alkyl aniline metabolites on the UROtsa cells have been examined so far. Considering that the urothelium is one of the targets of aromatic amine-induced carcinogenesis, we conducted this study to evaluate the protective effects of inorganic selenium (sodium selenite, SS) and organic selenium (selenomethionine, SM) against the cytotoxic, oxidant, and genotoxic properties of 3,5-DMAP in UROtsa cells. Additionally, we assessed the protective effects of ascorbic acid (Asc) and *N*-acetylcysteine (NAC) against the cytotoxic and ROS-inducing action of 3,5-DMAP.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals, including SS, SM, and the protease inhibitor cocktail, were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell culture reagents were purchased from Lonza (Walkersville, MD, USA) or Sigma-Aldrich. Molten normal melting point agarose, low melting point agarose, and fluorescence dyes SYBR Gold and Hoechst 33258 were purchased from Molecular Probes/Invitrogen (Eugene, OR, USA). GelBond film was obtained from Lonza. Bottomless 96-well plates for the comet assay were purchased from Greiner BioOne (Monroe, NC, USA).

Kits

Nuclear and cytoplasmic extraction kits were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Cell proliferation reagent (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) (WST-1) kit was purchased from Roche Applied Science (Indianapolis, IN, USA). 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFA) ROS detection kit was from Molecular Probes/Invitrogen. All other assay kits were from Sigma-Aldrich, except the thiobarbituric acid reactive substance (TBARS) and carbonyl assay kits (Cayman Chemical Company, Ann Arbor, MI, USA).

Synthesis of 3,5-dimethyaminophenol

3,5-DMAP was synthesised in our lab as described by Chao et al. (12). Briefly, a mixture of sulphanilic acid, sodium carbonate, and water was heated to 60 °C and then cooled in an ice bath. Sodium nitrite solution was added, and the resulting solution was poured on HCl solution (aqueous, 5.6% w/v) in an ice bath and kept for 15 min. This solution was added to 2,6-dimethylphenol solution (in aqueous NaOH and water) at 5 °C. The dark red reaction mixture was stirred well and kept at 25 °C for 1 h. Later, it was heated to 60 °C, and aqueous sodium hydrosulphite (1% w/v) was gradually added till yellow crystals were precipitated. After waiting for 15 min at 50 °C, the yellow suspension was cooled to 20 °C and then filtered. The filtrate was then washed with sodium hydrosulphite (1% w/v in water) and dried under vacuum. The final yield of 3,5-DMAP was 82%.

Cell culture and treatment

Cells of the UROtsa line were obtained from Thermo Fischer Scientific. UROtsa line is a primary culture of normal human urothelium isolated through immortalisation with a construct containing the SV40 large T antigen. In Dulbecco's Modified Eagle's Medium (DMEM) cells were grown as a monolayer in plastic flasks, containing foetal bovine serum (FBS, 5%, v/v) and glucose (1 mg/mL). The

cells were fed on fresh medium every three days and grown until reaching about 90% confluence. Confluent cultures were subcultured at a 1:4 ratio in a humidified atmosphere with 5% CO_2 at 37 ºC. Prior to all experiments, UROtsa cells were cultured with a 1:1 mixed serum-free medium of DMEM and Ham's F-12, supplemented with insulin (5 µg/mL), hydrocortisone (36 ng/mL), and EGF (10 ng/mL) for two days. For the experiments UROtsa cells (10^6) were plated in plastic flasks. After exposure, they were trypsinised and held at -80 °C until preparation of cytoplasmic and nuclear fractions. Cells exposed to 3,5-DMAP for 1 h were washed twice with fresh serum-free medium and then incubated in a fresh medium at 37 °C for another 24 h.

The experimental design is described in Table 1. Our preliminary 3,5-DMAP cytotoxicity experiments on the UROtsa cells included a wide dose range (1–1000 µmol/L) for several reasons. One was to determine the half-maximal inhibitory concentration (IC_{50}) for cell viability, and the other was to follow producers' kit protocol recommendations for one-hour exposure in the ROS experiments. The IC_{50} was determined to be 100 µmol/L, IC_{70} 50 µmol/L, and IC_{80} 25 µmol/L. As the IC_{80} (25 µmol/L) did not cause significant effects on oxidative stress parameters on the UROtsa cells, we selected the IC_{70} dose (50 µmol/L) for all subsequent experiments.

The Asc and NAC (±DMAP) treated groups were used for cytotoxicity and ROS assessments, while the SS and SM (±DMAP) groups were used for cytotoxicity, ROS, antioxidant parameter, apoptosis, and genotoxicity measurements.

The concentrations of SS and SM were chosen from preliminary experiments considering maximum glutathione peroxidase 1 (GPx1) induction (20–25). They were in the same ranges that were applied previously for several other cell types. The concentration of Asc and NAC was chosen based on our previous studies (10–12).

Exposure times of 24 and 72 h were also based on our previous studies with 3,5-DMAP (13, 14). Because the results obtained from the 24- and 72-hour treatment groups of both SS and SM were similar in most parameters measured, only the 24-hour results are included in the Results section.

Nuclear and cytoplasmic extract preparation

After centrifugation at 500 × *g* for 5 min, cell pellets were washed with PBS and re-centrifuged at 500 × *g* for 3 min. The supernatant was discarded and the obtained dry pellet used to obtain nuclear and cytoplasmic cellular fractions using a nuclear and cytoplasmic extraction kit (in the presence of a protease inhibitor cocktail).

Determination of cytotoxicity

The cytotoxicity assay was performed with a WST-1 kit. After treatment, the cells were washed with PBS, suspended in 1 mL of fresh medium, and then pipetted into 96-well plates. WST-1 was added to each well, and the cells incubated in the dark at 37 °C for 1 h.

Formazan absorbance was quantified with a spectrophotometer (SpectraMax® Microplate Reader, Molecular Devices, San Francisco, CA, USA) at 495 nm and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). ROS production in the cells was adjusted to negative control (assuming their ROS production as 100%).

Quantification of intracellular ROS

Intracellular ROS was detected with a CM-H_2DCFA ROS detection kit, which dyes living cells in fluorescent green.

The cells of either group were washed with PBS and suspended in 1 mL wells with serum-free medium. Cell suspension solutions were pipetted into 96-well plates and mixed with DMEM containing CM-H_2DCFDA (final concentration in each well: 25 µmol/L), activated by pre-incubation at 37 °C for 30 min. Generated ROS was detected immediately on a SpectraMax® Microplate Reader ($\lambda_{excitation}$:

Table 1 Experimental design

Experimental groups of UROtsa cells	Pretreatment	Treatment
Control #	-	-
SS		SS (30 nmol/L) for 24 h
SM		SM (10 µmol/L) for 24 h
DMAP		3,5-DMAP (50 µmol/L) for 1 h*
SS/DMAP	SS (30 nmol/L) for 24 h	3,5-DMAP (50 µmol/L) for 1 h*
SM/DMAP	10 µmol/L SM for 24 h	3,5-DMAP (50 µmol/L) for 1 h*
Asc		Asc (50 mg/mL) for 24 h
Asc/DMAP	Asc (50 mg/mL) for 24 h	3,5-DMAP (50 µmol/L) for 1 h*
NAC		NAC (5 mmol/L) for 24 h
NAC/DMAP	NAC (5 mM) for 24 h	3,5-DMAP (50 µmol/L) for 1 h*

Control – UROtsa cells with 90% confluence; SS – sodium selenite; SM – selenomethionine; DMAP – 3,5 dimethyaminophenol; Asc – ascorbic acid; NAC – N-acetylcysteine; * serum-free medium; The choice of the IC_{70} dose of 3,5-DMAP (50 µmol/L) was based on preliminary cytotoxicity experiments conducted on a wide dose range (1–1000 µmol/L)

485 nm, $\lambda_{emission}$: 530 nm) and quantified with SoftMax Pro software. ROS production was adjusted to negative control.

Determination of antioxidant enzyme activities

The activity of GPx1, a selenoenzyme mediating lipid peroxide and H_2O_2 reduction was measured with a respective Sigma Aldrich kit (see above) (18). TrxR activity was determined with an activity assay kit which can measure the reduction of the substrate 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) at 412 nm (19). Catalase (CAT) activity was determined with a colorimetric assay kit by measuring the enzymatic decomposition of H_2O_2 at 520 nm. Total superoxide dismutase (SOD) activity was measured with a total SOD activity kit. The inhibition of SOD activity was determined colorimetrically by measuring the reduction in the color development at 440 nm.

Glutathione reductase (GR) activity was determined with a GR activity assay kit. This assay mainly depends on the reduction of GSSG in the presence of NADPH, DTNB, and GR. Glutathione-S-transferase activity was determined with an assay kit using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate at 340 nm according to Habig et al. (26).

Determination of total, reduced, and oxidised glutathione levels

Total glutathione (GSH) concentrations of different cellular compartments were determined with a total glutathione assay kit. For the quantification of GSSG, reduced GSH was blocked by the addition of 2-vinylpyridine in the presence of triethanolamine. GSH concentrations were calculated using the following equation: GSH = (total GSH-2xGSSG). Cytoplasmic and nuclear redox ratio ([GSH]/[GSSG]) was calculated by dividing the GSH concentration with the GSSG concentration.

Determination of lipid peroxidation

Lipid peroxidation in nuclear and cytoplasmic extracts was quantified by measuring the concentration of thiobarbituric acid reactive substance (TBARS) spectrofluorometrically using a TBARS assay kit (27), as it reflects malondialdehyde (MDA) concentrations, and the results were expressed as μmol/g of protein.

Determination of protein oxidation

The most commonly used indicator of protein oxidation is protein carbonyl content (28), which was measured with a carbonyl assay kit at 360 nm spectrophotometrically.

Total protein determination

Protein content of the samples was determined with the bicinchoninic acid (BCA) protein assay kit. The absorbance values of the samples were measured at 562 nm, and the results expressed as mg/mL (29).

Determination of caspase 3 and caspase 8 activities

Caspase 3 activity was measured with a colorimetric assay kit based on hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (AcDEVD-pNA), which results in release of the p-nitroaniline (pNA) moiety. Total pNA released from the substrate was calculated from the absorbance values at 405 nm or from a calibration curve obtained from pNA standards.

Caspase 8 activity was also measured with a colorimetric assay kit based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETDpNA), which results in the release of a pNA moiety. Total pNA released from the substrate was determined from the absorbance values at 405 nm or from a standard pNA calibration curve.

Comet assay

Comet assay is a sensitive, fast, and flexible method that can easily detect DNA damage caused by environmental chemicals on different cell types. It is also useful in evaluating the ability of antioxidants/micronutrients to protect the integrity of the genetic material (30). The CometChip protocol, first described by Wood et al. (31), is gaining significant importance for high throughput DNA damage analysis.

Single strand breaks (SSBs) were determined using the alkaline CometChip protocol, while double strand breaks (DSBs) were detected using the neutral CometChip protocol. Briefly, for SSB determination, treated UROtsa cells (100 μL, 10^6 cells/mL) were pipetted into a bottomless 96-well plate and captured by gravity. The plate was then removed and the gel covered with 1% low melting point agarose. The cells were loaded to CometChips and lysed in a lysis buffer. This buffer consisted of 0.3 mol/L NaOH, 2.5 mol/L NaCl, 10 mmol/L Tris, 1% Triton X, and 1 mmol/L Na_2EDTA. After overnight lysis, they were electrophoresed in a cold alkaline electrophoresis buffer (0.3 mol/L NaOH and 1 m mol/L Na_2EDTA) at 4 °C for 40 min. The chips were then neutralised twice in fresh buffer (0.4 mol/L Tris-HCl at pH 7.5) at 4 °C for 15 min and stained with SYBR Gold according to the manufacturer's instructions for fluorescence imaging. Images were captured using a Nikon 80i upright microscope (Nikon Instruments, Tokyo, Japan) coupled with an automatic scanning system and analysed with a customised Guicometanalyzer software by MATLAB (The Mathworks, Natick, MA, USA).

For DSB determination, the gels were lysed in a neutral lysis buffer [2.5 mol/L NaCl, 2 mmol/L Na_2EDTA, 10 mmol/L Tris, 1% *N*-lauroylsarcosine, pH 9.5 with 0.5% Triton X-100, and 10% dimethyl sulphoxide (DMSO) added 20 min before use] at 43 °C for 4 h. Chips were then washed three times with the electrophoresis buffer (90 mmol/L Tris, 90 mmol/L boric acid, and 2 mmol/L Na_2EDTA, pH 8.5) for 30 min and electrophoresed at 4 °C for 1 h. After neutralisation, the CometChips were stained with SYBR

Gold for fluorescence imaging. Images were captured and analysed in the same manner as with the alkaline CometChip protocol. For DNA damage parameters we took %tail DNA as the level of DNA damage and olive tail moment (OTM, which represents the product of comet length and tail intensity).

Untreated cells were used as controls, cells treated with H_2O_2 (100 µmol/L) as positive control, and cells treated with 1% DMSO as solvent control. All experiments were done in at least triplicate for each group.

Statistical analysis

The results obtained from triplicate measurements were expressed as mean ± standard deviation (SD) and analysed with the Statistical Package for Social Sciences Program (SPSS) version 17.0 (Chicago, IL, USA) to compare the study groups. Differences between the groups were evaluated with the Kruskal-Wallis one-way analysis of variance (ANOVA) and later with the Mann Whitney U-test. P values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity and ROS production caused by 3,5-DMAP and protection by antioxidants

Treatment of UROtsa cells with SS, SM, Asc, or NAC alone caused no detectable cytotoxicity, whereas 3,5-DMAP caused a dose-dependent increase in cytotoxicity.

All antioxidants protected the cells against cytotoxicity caused by 3,5-DMAP (Figure 1). 3,5-DMAP significantly increased intracellular ROS generation, and all antioxidants lowered it significantly. Asc and NAC provided almost full protection, and SS and SM only partial protection (Figure 2). At the IC_{50} of 3,5-DMAP, SS pretreatment restored cell viability to 100%, and SM increased it to 95%. All the oxidant and antioxidant parameters were also detected in the nucleus but were two to nine times lower than in the cytoplasm.

Similar protective properties of SS and SM against the cytotoxic and ROS-producing effects of 3,5-DMAP were also found in AS52 CHO cells. However, the IC_{50} of 3,5-DMAP in AS52 CHO cells was one quarter of ours, 25 µmol/L (13, 14). Considering our previous findings, we believe that UROtsa cells are more resistant to the cytotoxic effects of 3,5-DMAP, perhaps because the UROtsa cells are derived from primary cells and then immortalised. However, even though human urothelial cells have a higher IC_{50} than different strains of CHO cells, in real life urothelial cells are exposed to many other chemicals and their metabolites besides alkyl aniline metabolites, and their combined effects may lead to carcinogenic changes. These combined effects should be investigated in future studies.

Selenoenzymes

Selenoenzymes, GPxs in particular, protect cells from oxidative damage caused by peroxides (18). TrxRs are responsible for the reduction of thioredoxin, which is a very

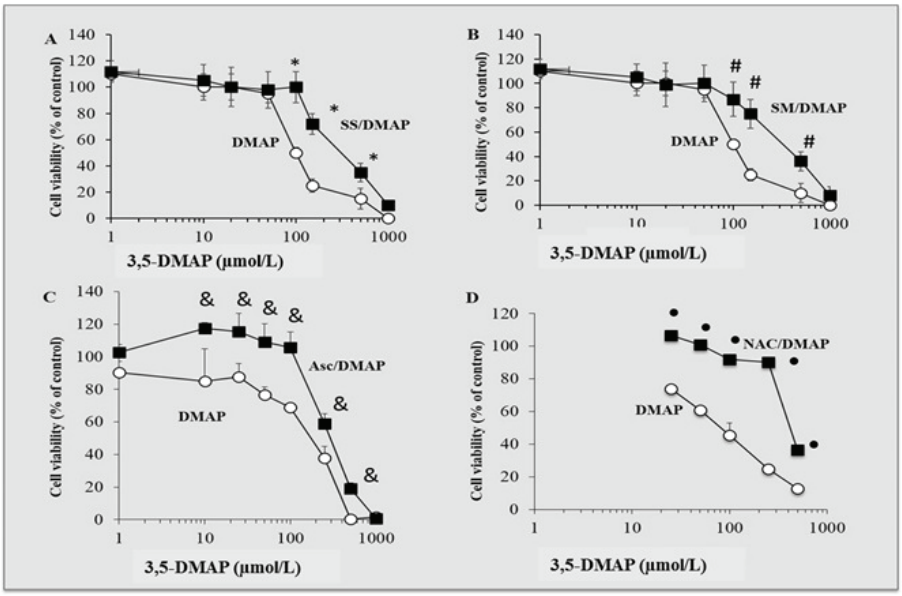

Figure 1 Cell viability expressed in the percentage of control. Values are given as means ± SD of duplicate measurements of three flasks per group; 3,5-DMAP – 3,5-dimethylaminophenol; SM – selenomethionine; SS – sodium selenite; A – cell viability after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with SS for 24 h and then treated with 3,5-DMAP for 1 h (SS/DMAP); B – cell viability after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with SM for 24 h and then with 3,5-DMAP for 1 h (SM/DMAP); C – cell viability after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with ascorbic acid (Asc) for 24 h and then with 3,5-DMAP for 1 h (Asc/DMAP); D: cell viability after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with *N*-acetylcysteine (NAC) for 24 h and then with 3,5-DMAP for 1 h (NAC/DMAP); $^{*,\#,\&,\cdot}$ and $^{\cdot}$ – significantly higher than DMAP ($p<0.05$)

Table 2 Cytoplasmic and nuclear selenoenzyme activities in UROtsa cells

	Cytoplasmic		Nuclear	
	GPx1 (U/mg protein)	TrxR (mU/mg protein)	GPx1 (U/mg protein)	TrxR (mU/mg protein)
Control	0.221±0.012	0.468±0.012	0.056±0.001	0.0660.003
SS	0.347±0.015*	0.691±0.056*	0.071±0.004*	0.098±0.005*
SM	0.326±0.029*	0.698±0.061*	0.067±0.002*	0.091±0.011*
DMAP	0.127±0.018*,#,&	0.316±0.042*,#,&	0.034±0.001*,#,&	0.041±0.010*,#,&
SS/DMAP	0.199±0.016	0.567±0.048	0.052±0.002	0.065±0.005
SM/DMAP	0.213±0.019	0.521±0.064	0.058±0.003	0.059±0.012

DMAP – 3,5-dimethylaminophenol (one-hour treatment with the IC_{70} dose of 50 µmol/L); GPx1: glutathione peroxidase 1; SS – 24-hour pretreatment with sodium selenite; SM – 24-hour pretreatment with selenomethionine; TrxR – thioredoxin reductase; Values are given as means ± SD of duplicate measurements of three flasks per group; * significantly different from control ($p<0.05$); # significantly different from the SS/DMAP group ($p<0.05$); & significantly different from the SM/DMAP group ($p<0.05$)

important protein in many biological processes, including redox signalling (19). Cytoplasmic and nuclear selenoenzyme activities are summarised in Table 2. As expected, both SS and SM treatments significantly increased GPx1 and TrxR activities in both the cytoplasm and nucleus. 3,5-DMAP treatment, in turn, lowered them significantly. Combined SS and SM treatment with 3,5-DMAP resulted in significant restoration of cytoplasmic and nuclear GPx1 and TrxR activities to nearly control levels.

These and similar drops in GPx1 and TrxR activities in our previous studies may be related to lower expression of these antioxidant proteins but also to post-translational modification caused by 3,5-DMAP exposure. Further mechanistic studies are needed to investigate post-transcriptional or post-translational effects of alkyl anilines and their metabolites on selenoproteins. Moreover, selenoprotein P expression/level should also be measured *in vivo* after applying 3,5-dimethylaniline, as it is the most important antioxidant selenoprotein in eukaryotic plasma, epithelial, and endothelial cells.

Antioxidant enzymes

One-hour exposure to 50 µmol/L of 3,5-DMAP exposure led to substantial changes in cellular antioxidant/oxidant parameters in UROtsa cells, indicating a shift in the redox equilibrium towards oxidation. Table 3 shows the activities of cytoplasmic and nuclear antioxidant enzymes.

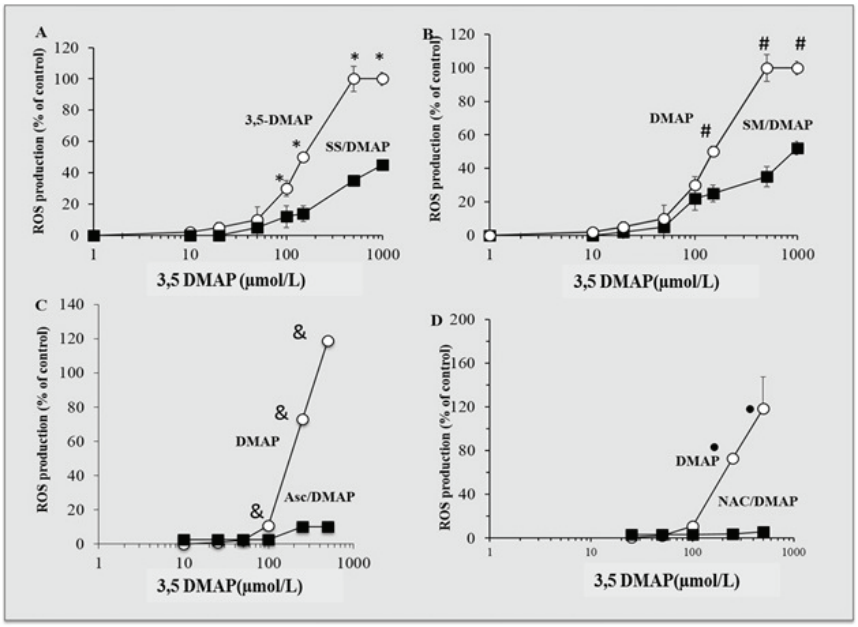

Figure 2 Intracellular ROS production expressed in the percentage of control. Values are given as means ± SD of duplicate measurements of three flasks per group; 3,5-DMAP – 3,5-dimethylaminophenol ; SM – selenomethionine; SS – sodium selenite; A – ROS production after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with SS for 24 h and then treated with 3,5-DMAP for 1 h (SS/DMAP); B: ROS production after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with SM for 24 h and then with 3,5-DMAP for 1 h (SM/DMAP); C: ROS production after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with ascorbic acid (Asc) for 24 h and then with 3,5-DMAP for 1 h (Asc/DMAP); D: ROS production after exposure to 3,5-DMAP for 1 h (DMAP) or in the group pretreated with *N*-acetylcysteine (NAC) for 24 h and then with 3,5-DMAP for 1 h (NAC/DMAP); *, #, &, and ˙ – significantly lower than DMAP ($p<0.05$)

Table 3 Cytoplasmic and nuclear antioxidant enzyme activities in UROtsa cells

	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	0.40±0.05	30.24±2.338	0.148±0.01	39.15±2.18	0.14±0.01	10.24±1.03	0.090.01	5.91±0.29
SS	0.55±0.06*	45.25±2.359*	0.152±0.02	40.12±2.07	0.13±0.01	11.01±0.9	0.080.01	5.12±0.61
SM	0.54±0.06*	48.72±3.025*	0.161±0.03	41.12±3.01	0.13±0.02	11.32±	0.100.02	5.29±0.59
DMAP	0.30±0.04*$^{\#,\&}$	61.11±2.729*$^{\#,\&}$	0.082±0.02*$^{\#,\&}$	28.14±1.98*$^{\#,\&}$	0.09±0.02*$^{\#,\&}$	15.13±1.16*$^{\#,\&}$	0.07±0.01*$^{\#,\&}$	3.07±0.38*$^{\#,\&}$
SS/DMAP	0.51±0.03*	32.18±1.358	0.139±0.01	34.18±1.15	0.12±0.02*	12.12±1.18	0.10±0.01	6.01±0.18
SM/DMAP	0.50±0.04*	33.18±1.862	0.141±0.02	35.99±1.08	0.11±0.01*	13.09±2.01	0.110.02	5.59±0.87

CAT – catalase; SOD – total superoxide dismutase; GR – glutathione reductase; GST – glutathione S-transferase; DMAP – 3,5-dimethylaminophenol (one-hour treatment with the IC_{70} dose of 50 µmol/L); SS – sodium selenite; SM – selenomethionine; Values are given as means ± SD of duplicate measurements of three flasks per group; *significantly different from control ($p<0.05$); # significantly different from the SS/DMAP group ($p<0.05$); & significantly different from the SM/DMAP group ($p<0.05$)

3,5-DMAP significantly lowered both cytoplasmic and nuclear GPx1 and TrxR activities, which was counteracted by SS and SM treatment (Table 3). The same phenomenon was also observed in AA8 and AS52 CHO cells (13, 14).

SS and SM treatment significantly increased the cytoplasmic, but not nuclear CAT and SOD activities. DMAP exposure significantly decreased cytoplasmic CAT (35%), GR (45%), and GST (28%) activities and caused a significant (twofold) increase in cytoplasmic SOD activity. 3,5-DMAP treatment also significantly decreased nuclear CAT (36%), GR (22%), and GST (48%) activities. Nuclear SOD activity dropped 1.5 times ($p<0.05$).

Almost the same results were previously found in AA8 and AS52 CHO cells (12-14), which clearly points to 3,5-DMAP causing an imbalance between cellular enzymatic antioxidants and cellular oxidants in different eukaryotic cells. This needs to be confirmed by future *in vivo* experiments, which should also establish the expression of enzymatic antioxidants.

Total, oxidised, and reduced glutathione levels

Cellular thiols, GSH in particular, play an important role in maintaining cellular redox status (32). The effects of 3,5-DMAP (50 µmol/L) on total, oxidised, and reduced GSH levels and intracellular redox ratios are summarised in Table 4. DMAP significantly lowered total GSH levels in the cytoplasm compared to control. In the SS/DMAP and SM/DMAP treatment groups (data not shown for 72 h groups because there were no differences between 24-hour and 72-hour treatment) total GSH levels were significantly higher than in the DMAP alone group. 3,5-DMAP increased cytoplasmic GSSG levels 2.3 times ($p<0.05$). In cells pretreated with SS and SM they dropped significantly but not to control levels. The cytoplasmic redox ratio was significantly higher in the groups treated with SS and SM alone than in controls ($p<0.05$). 3,5-DMAP decreased the redox ratio 2.7 times. Nuclear total GSH levels increased significantly in the SS and SM groups and decreased significantly (1.6 times) in the DMAP group. In the SS/DMAP and SM/DMAP groups, total GSH levels were significantly higher than in the DMAP group.

Due to decreases in total GSH levels in the DMAP alone group, nuclear GSSG levels were lower than control ($p<0.05$). Nuclear redox ratio was significantly higher in the SS and SM groups. 3,5-DMAP lowered the ratio 2.7 times *vs*. control while pretreatment with SS and SM restored the ratio to control levels.

Lipid peroxidation and protein oxidation

Data regarding lipid peroxidation (TBARS) and protein oxidation are also given in Table 4. Cytoplasmic lipid peroxidation levels were 6.6 times higher in the DMAP group (50 µmol/L) *vs*. control ($p<0.05$). Pretreatment with SS and SM decreased lipid peroxidation significantly, but cytoplasmic lipid peroxidation levels in the SS/DMAP and

Table 4 Glutathione (total, oxidised, reduced) levels, redox ratio, lipid peroxidation, and protein oxidation levels in UROtsa cells

	Cytoplasmic					Nuclear					
	Total GSH (nmol/$mg_{protein}$)	GSSG (nmol/$mg_{protein}$)	GSH (nmol/$mg_{protein}$)	Redox Ratio	TBARS (µmol/$g_{protein}$)	Carbonyl (µmol/$mg_{protein}$)	Total GSH (nmol/$mg_{protein}$)	GSSG (nmol/$mg_{protein}$)	GSH (nmol/$mg_{protein}$)	Redox Ratio	TBARS (µmol/$g_{protein}$)	Carbonyl (µmol/$mg_{protein}$)
Control	22.12±1.12	2.98±0.34	16.16±0.78	7.42	0.16±0.03	2.14±0.21	7.21±0.72	0.74±0.22	5.73±0.51	9.74	0.06±0.01	1.12±0.11
SS	23.15±1.56	2.41±0.21	18.33±0.69	9.61*	0.14±0.02	2.21±0.13	7.88±0.21	0.56±0.12*	6.76±0.15	14.07*	0.05±0.00	1.13±0.05
SM	24.11±2.01	2.57±0.16	18.97±1.02	9.38*	0.13±0.03	2.18±0.22	7.54±0.41	0.59±0.08*	6.36±0.24	12.78	0.05±0.01	1.05±0.08
DMAP	18.54±1.16*,#,&	6.77±1.01*,#,&	5.00±1.05*,#,&	2.74*,#,&	1.05±0.02*,#,&	4.05±0.24*,#,&	4.52±0.28*,#,&	0.52±0.11*,#,&	3.48±0.17*,#,&	6.28*,#,&	0.09±0.01*,#,&	2.12±0.10*,#,&
SS/DMAP	19.87±1.21	5.02±0.57*	9.83±0.76*	3.96*	0.75±0.01*	3.12±0.12*	7.01±0.15	0.65±0.12	5.71±0.13	10.78	0.05±0.01	1.88±0.08*
SM/DMAP	19.42±1.18	4.99±0.39*	9.44±0.73*	3.89*	0.78±0.02*	3.05±0.15*	6.84±0.37	0.69±0.09	5.46±0.25	9.91	0.05±0.01	1.75±0.09*

GSH – reduced glutathione; total GSH – total glutathione; GSSG – oxidised glutathione; DMAP – 3,5-dimethylaminophenol (one-hour treatment with the IC_{70} dose of 50 µmol/L); SS – sodium selenite; SM – selenomethionine; TBARS – thiobarbituric acid reactive substance; Values are given as means ± SD of duplicate measurements of three flasks per group; *significantly different from control ($p<0.05$); # indicates that the mean is significantly different from the SS/DMAP group ($p<0.05$); & indicates that the mean is significantly different from the SM/DMAP group ($p<0.05$)

SM/DMAP groups were still significantly higher than in control (4.7 and 4.8 times, respectively). Nuclear lipid peroxidation was 1.5 times higher in the DMAP group than control ($p<0.05$). In the SS/DMAP and SM/DMAP it was restored to control levels.

Increased lipid peroxidation is known to facilitate protein oxidation. The best biomarker of protein oxidation is protein carbonylation (33). However, protein oxidation can arise independently (33, 34), and protein peroxides can induce lipid oxidation *via* radical-mediated reactions (33–35). We found that cytoplasmic and nuclear protein oxidation increased significantly in 3,5-DMAP treated cells as evidenced by increases in protein carbonyl levels (Table 4). 3,5-DMAP treatment increased cytoplasmic carbonyl levels (indicator of protein oxidation) 1.9 times compared to control. Pretreatment with SS and SM reduced cytoplasmic protein oxidation, but it remained significantly higher than in control (1.5 times in the SS/DMAP group and 1.4 times higher in the SM/DMAP). Nuclear carbonyl levels were also 1.9 times higher in the DMAP group than control cells, and remained significantly higher in the SS/DMAP and SM/DMAP groups (1.7 and 1.6 times, respectively).

Our results suggest that protein carbonyl levels increased due to increases in lipid peroxidation. Similar results were reported for CHO AS52 and AA8 cells (12-14). All these studies clearly show that increases in lipid peroxidation may significantly lead to protein oxidation (33–35), and oxidised proteins lose their ability to interact with cellular processes, which may also lower the activities of enzymatic antioxidants.

CometChip

Overproduction of ROS and increased lipid peroxidation combined with insufficient antioxidant enzymes causes different types of DNA damage (36). Even though the major DNA lesions are caused by direct oxidation of DNA bases, modifications caused by lipid peroxidation products, MDA in particular, may damage DNA nearly as much as direct base oxidation. MDA reacts with guanine, adenine, and cytosine to form cyclic pirymido-[1,2α] purine-10(3H)-one-2′-deoxyribose (M1dG) adduct, the major MDA-DNA adduct in rodent and human tissues. M1dG induces transversions (particularly to T) and transitions (especially to A) with a frequency comparable with that of 8-oxoG in *Escherichia coli* (35, 36).

Figure 3 shows DNA damage induced by 3,5-DMAP (50 µmol/L). Neither SS nor SM alone reduced the olive tail moment (OTM), whereas 3,5-DMAP significantly increased both the %tail DNA (25%) and OTM (24%) compared to control. Pretreatment with SS and SM significantly lowered %tail DNA (24% and 26%, respectively) as well as OTM (17% and 20%, respectively). The neutral comet assay did not reveal any changes in %tail

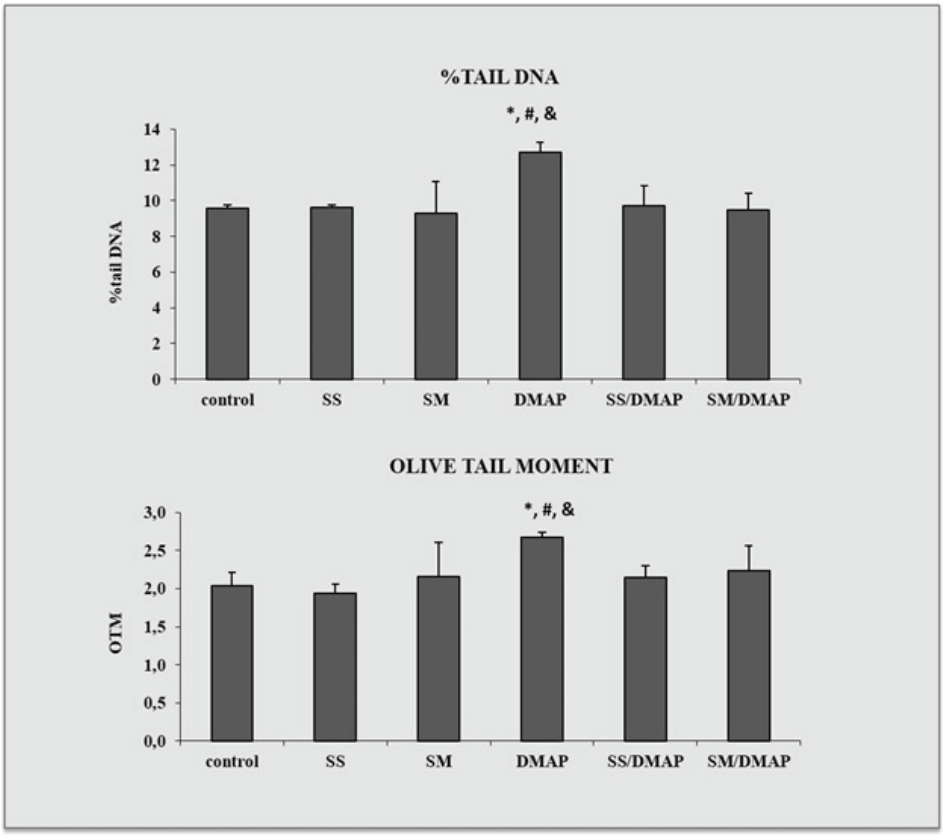

Figure 3 %tail DNA and OTM values obtained with the alkaline CometChip assay; OTM – olive tail moment; DMAP – 3,5-dimethylaminophenol (IC_{70} dose of 50 μmol/L); SM – selenomethionine; SS – sodium selenite; * significantly higher than in control; # significantly higher than in SS/DMAP; & significantly higher than in SM/DMAP

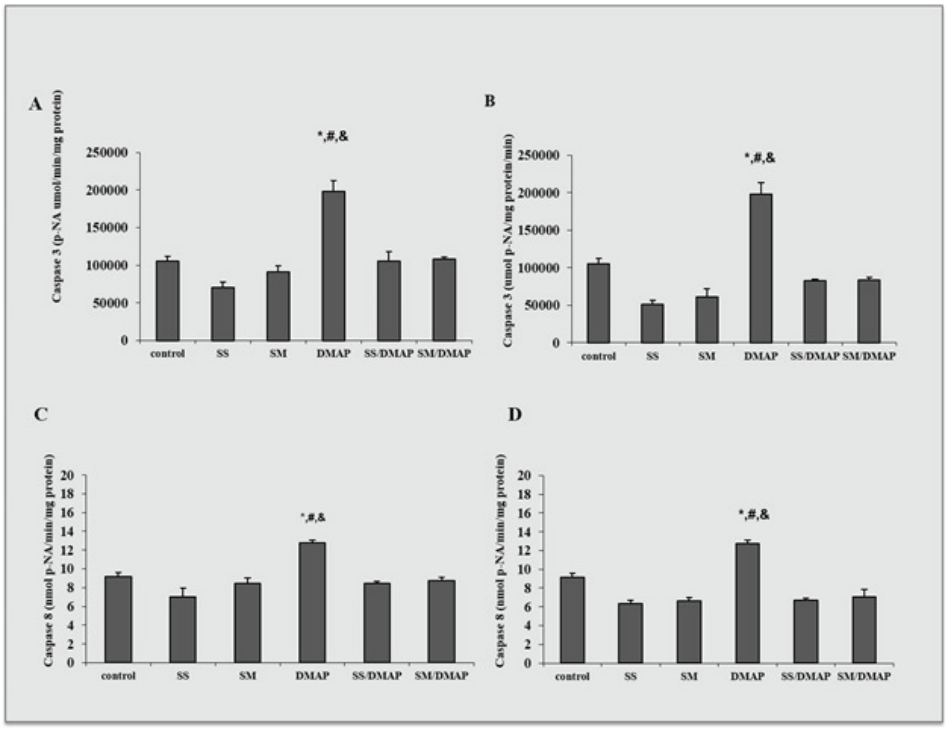

Figure 4 Caspase 3 and caspase 8 activities in the study groups. Values are given as means ± SD of duplicate measurements of three flasks per group; A – Caspase 3 activity in the groups pretreated with SS or SM for 24 h; B – Caspase 3 activity in the groups pretreated with SS or SM for 72 h; C – Caspase 8 activity in the groups pretreated with SS or SM for 24 h; D – Caspase 8 activity in the groups pretreated with SS or SM for 72 h); DMAP – 3,5-dimethylaminophenol (IC_{70} dose of 50 μmol/L); SM – selenomethionine; SS – sodium selenite; * significantly different from control; # significantly different from SS/DMAP; & significantly different from SM/DMAP

DNA and OTM after 3,5-DMAP treatment, perhaps because of its lower sensitivity to the alkaline comet assay.

Significantly increased %tail DNA and OTM in the 3,5-DMAP-treated cells confirm its damaging potential in the DNA. Our previous study (14) showed a similar decrease in DNA damage in CHO AS52 cells pretreated with SS and SM. Other studies also confirm their protective effect against DNA damage induced by a variety of compounds, including phthalates.

The results of CometChip clearly show that 3,5-DMAP causes DNA damage, possibly through the oxidation of DNA bases. Studies on a wider spectrum (with mechanistic data) than ours should be able to show what types of DNA damage 3,5-DMAP exposure can cause. As most of the DNA damage is related to the oxidation of guanine (8-oxodeoxyguanine formation), measurements of DNA repair protein levels [8-oxoguanine glycosylase (OGG1) in particular] should be able to show how 3,5-DMAP affects the base excision repair pathway.

Caspase 3 and 8 activities

Caspases are a family of protease enzymes playing essential roles in apoptosis. Caspase 8 is an initiator caspase that leads to the activation of caspase 3 as an executioner caspase. Figure 4 summarises cellular caspase 3 and caspase 8 activities in our study. 3,5-DMAP caused a significant (87%) increase in caspase 3 activity, while pretreatment with SS and SM lowered it 46% and 45% ($p<0.05$), respectively in the first 24 h. After 72 h SS lowered it 58% and SM 57%.

Caspase 8 activity rose 39% in the DMAP group in respect to control, while pretreatment with SS lowered it 33% and with SM 31% after 24 h compared to DMAP alone ($p<0.05$). After 72 h, SS pretreatment also resulted in a significant decrease in caspase 8 activity. Due to a high standard deviation in the SM pretreatment group, this drop was not significant.

Apoptosis is characterised by lower cellular GSH content (37), and our findings of reduced GSH levels and apoptosis induction in 3,5-DMAP-treated cells confirm that association. They also confirm the protective effects of the tested selenocompounds against cell death caused by this alkyl aniline metabolite. Similar results were previously reported for CHO AA8 and AS52 cells (12–14).

Our results clearly point to 3,5-DMAP as the cause of apoptosis in human urothelial cells. However, other forms of cell death, autophagy in particular (as well as different autophagic markers), should also be investigated in order to determine the mechanism of cell death caused by alkyl anilines.

In conclusion, this is the first study that shows the toxic effects of 3,5-DMAP on human urothelial cells. As low selenium status is suggested to be one of the most important factors in the emerging of bladder cancer, protective effects of both organic and inorganic selenium were also evaluated. Both selenocompounds were found to be protective against the cytotoxic, genotoxic, and ROS-producing effects of 3,5-DMAP, which confirmed the importance of selenium status. Moreover, we have observed that 3,5-DMAP induced apoptosis can be prevented by selenium supplementation. Our future aim is to conduct *in vivo* studies that can provide more mechanistic information on the mode of action of 3,5-DMAP.

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Antioksidansi i spojevi selenija inhibiraju toksično djelovanje 3,5-dimetilaminofenola na epitelne bubrežne stanice u ljudi

Izloženost alkilnim anilinima može uzrokovati rak mokraćnoga mjehura, koji je drugi po redu po učestalosti raka mokraćno-spolnog sustava. 3,5-dimetilanilin često se rabi u industrijskoj proizvodnji, a istraživanja njegova primarnog metabolita, 3,5-dimetilaminofenola (3,5-DMAP), pokazuju da on uzrokuje oksidacijski stres i promjene u aktivnosti antioksidacijskih enzima te u konačnici dovodi do smrti raznih stanica u sisavaca. Dosad, međutim, nije provedeno nijedno istraživanje njegovih izravnih učinaka na epitelne stanice mokraćnoga mjehura i bubrega u ljudi. Za spojeve selenija smatra se da smanjuju oksidacijski stres različitih kemikalija te da dopuna prehrane selenijem smanjuje rizik od raka mokraćnoga mjehura. Primarni je cilj ovoga istraživanja bio utvrditi može li organski spoj selenija selenometionin (SM, 10 μmol/L), odnosno anorganski spoj natrijev selenit (SS, 30 nmol/L], smanjiti oksidacijski stres, oštećenje DNA i apoptozu u UROtsa stanicama izloženima 3,5-dimetilaminofenolu. Jednosatna izloženost stanica 3,5-DMAP-u dovela je do povećanja razina reaktivnih kisikovih spojeva (ROS), lipidne peroksidacije, oksidacije bjelančevina te do promjena u aktivnosti antioksidacijskih enzima u staničnoj citoplazmi i jezgri, ovisno o primijenjenoj dozi. Osim toga, komet-testom su utvrđeni jednolančani, ali ne i dvolančani lomovi DNA. Također, 3,5-DMAP uzrokovao je apoptozu stanica. Oba su spoja selenija pružila djelomičnu zaštitu od njegova toksičnoga djelovanja. Nedostatak selenija pri izloženosti alkilnim anilinskim spojevima stoga može odigrati značajnu ulogu u nastanku raka mokraćnog mjehura. Potrebna su daljnja istraživanja mehanizama djelovanja selenija u njegovu sprječavanju.

KLJUČNE RIJEČI: alkilni anilini; citotoksičnost; genotoksičnost; reaktivni kisikovi spojevi; natrijev selenit; selenij; selenometionin