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Original Contribution

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

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ABSTRACT

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



## Introduction

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

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

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

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

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

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

## Materials and methods

### *Chemicals and reagents*

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

### *Cell culture and treatment*

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

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

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

### *Determination of cell viability*

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

### *Antioxidant enzyme activities and glutathione levels*

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

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

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

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

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

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

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

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

### *Alkaline single-cell gel electrophoresis (comet assay)*

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

### *Statistical analysis*

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

## Results

### *Selenium supplementation increases resistance to DEHP and MEHP cytotoxicity*

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

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

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

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

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

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

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

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

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

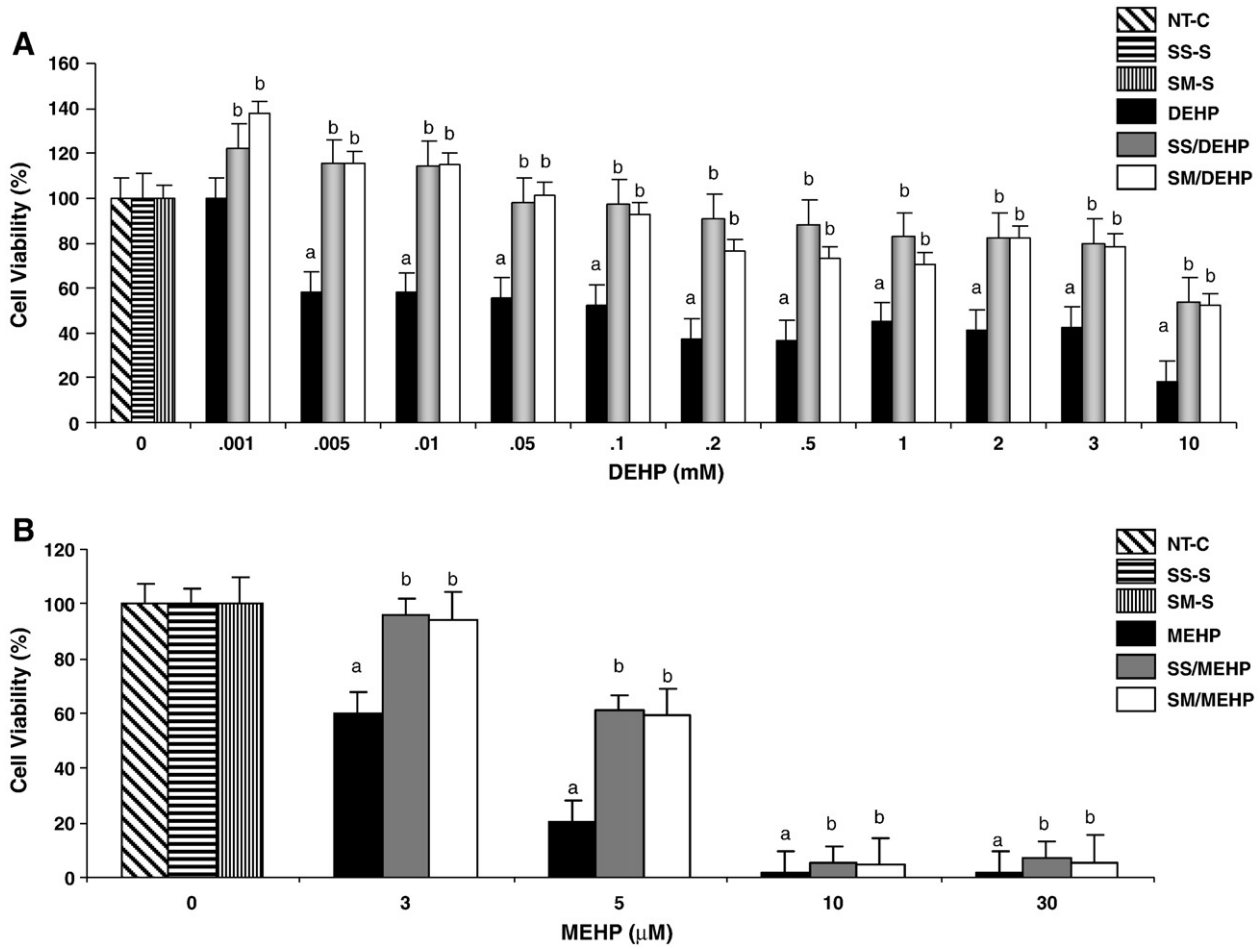


**Fig. 1.** Cell viability in phthalate-exposed LNCaP cell line, and protective effect of selenium supplementation. Cell viability was determined by the MTT assay and data were presented as relative to zero dose of DEHP or MEHP. Values are given as mean ± SEM of $n = 3$ experiments and triplicate measurements.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

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

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

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

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

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

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

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

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

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

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

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

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

## Discussion

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

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

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

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

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

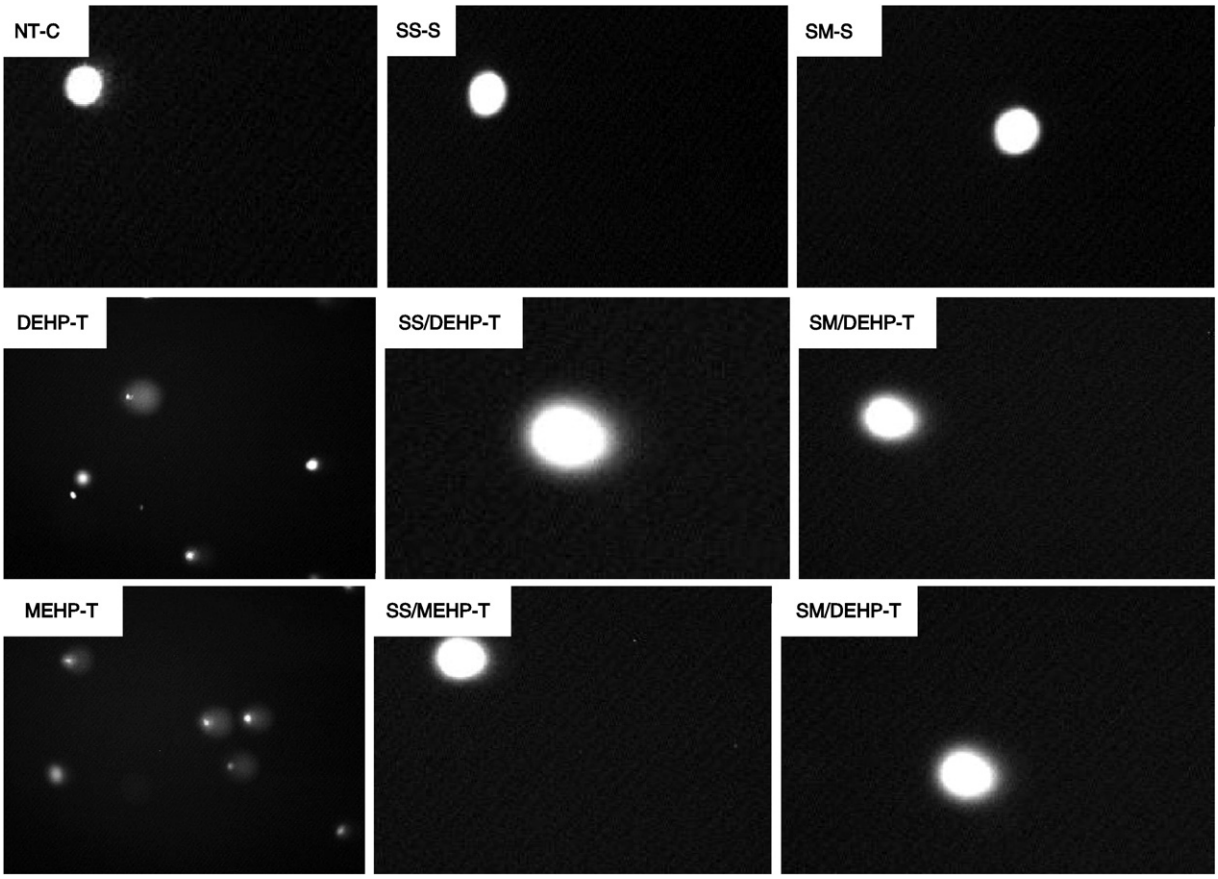


**Fig. 2.** Typical images of comets in DEHP- or MEHP-treated LNCaP cells and effects of selenium supplementation. Measurements were performed in the following treatment groups of cells: NT-C, nontreated LNCaP cells cultured for 24 h; SS-S, LNCaP cells supplemented and cultured with 30 nM SS for 72 h;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

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

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

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

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

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

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

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