# CONVENTIONAL CELL CULTURE MEDIA DO NOT ADEQUATELY SUPPLY CELLS WITH ANTIOXIDANTS AND THUS FACILITATE PEROXIDE-INDUCED GENOTOXICITY

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**Abstract**—Commercially available calf serum did not supply the cultured murine fibroblast cell line L929 with amounts of selenium and $\alpha$-tocopherol sufficient to protect against peroxide damage. Supplementation of the culture medium with 30 $\mu$M $\alpha$-tocopherol or 50 nM sodium selenite led to a substantial increase of cellular $\alpha$-tocopherol concentrations from 18 $\pm$ 3.0 to 3179 $\pm$ 93.0 pmol/$10^6$ cells or cellular selenium concentrations from 0.17 $\pm$ 0.02 to 1.75 $\pm$ 0.16 ng/$10^6$ cells, respectively. L929 fibroblasts grown in selenite-containing medium also had markedly raised activities of both cytosolic glutathione peroxidase (from 11 $\pm$ 0.9 to 67.2 $\pm$ 4.2 mU/$10^7$ cells) and phospholipid hydroperoxide glutathione peroxidase (from 0.2 to 9.5 $\pm$ 0.9 mU/$10^7$ cells). Supplementation with $\alpha$-tocopherol inhibited single-strand breaks induced by low concentrations of $H_2O_2$ only, whereas an adequate selenium supply almost completely inhibited single-strand breaks induced by up to 30 $\mu$M $H_2O_2$ and also significantly reduced $H_2O_2$-induced cell death. An inadequate selenium supply and corresponding increase of GPx activity upon selenite supplementation was also observed with other cell lines, for instance, D10N, ECV-304, HepG2, and THP-1. Our data strengthen the relevance of standardized and adequate supplementation of tissue culture media with antioxidants to improve viability and genetic stability of cultured cells in general and in particular, if they are oxidatively challenged.

**Keywords**—Cell culture, Selenium supply, Comet assay, DNA single strand breaks, Glutathione peroxidase, $\alpha$-Tocopherol, Free radicals

## INTRODUCTION

Cells usually contain a variety of different antioxidant systems in order to counteract the detrimental effects of reactive oxygen species.[1] In mammals or mammalian cell cultures several antioxidants and basic components of complex antioxidant systems have to be supplied by external, for instance, nutritional sources. Results from in vivo and in vitro studies suggesting that oxygen radicals act as cell- and DNA-damaging agents support the hypothesis that antioxidants can inhibit the initiation and exacerbation of reactive oxygen species (ROS) associated diseases and provide impetus for exploring the function of certain dietary antioxidants in the prevention of cancer,[2,3] atherosclerosis,[4,5] and neurodegenerative diseases.[6,7]

One of the substances that may have such a potential is $\alpha$-tocopherol. This radical chain-breaking lipid antioxidant protects biological membranes from the propagation of lipid peroxidation, thereby preventing the formation of secondary, highly reactive metabolites such as $OH^{\cdot}$, 4-hydroxy-nonenal or decadienals,[8,9] which are able to modify different cellular structures, including DNA. Although much of the oxidized tocopherol may be regenerated in the presence of other cellular antioxidants, there are also irreversible losses that lead to a depletion of cellular tocopherol pools unless substituted for by external sources. Selenium is another important factor for cellular antioxidant defense that needs continuous external supply. It does not act as an antioxidant by itself, but has to be incorporated into selenoproteins as selenocysteine.[10,11] The

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best-characterized selenoenzymes are the glutathione peroxidases (GPx).[12] Three of those, the intracellular types, for instance the cytosolic glutathione peroxidase (cGPx),[13,14] the phospholipid hydroperoxide glutathione peroxidase (PHGPx),[15,16] and the gastrointestinal glutathione peroxidase[17] may protect cells from hydroperoxide toxicity by their ability to reduce peroxides to alcohols in the presence of glutathione.

Cell lines cultured for extended periods of time usually have the bovine serum supplemented to culture medium as only source of selenium or $\alpha$-tocopherol. Nevertheless, such cell lines with generally undefined antioxidant status are used to study the toxicity and mutagenicity of ROS-generating substances or pathways of signalling subject to redox regulation. In the present study we, therefore, examined whether the precultivation of murine L929 fibrosarcoma cells in medium containing small supplements of dietary antioxidants may influence adverse ROS effects towards this cell line which is commonly used for experiments involving ROS generation. In order to challenge the cells we chose low concentrations of $H_2O_2$, as they are produced, for example, in an inflammatory setting or after endotoxin exposure. As a read-out system for genotoxicity the recently developed comet assay[18–20] was chosen. Further, the cells were subjected to various viability tests.

We found that cells grown in conventional media are severely deficient in cellular antioxidant devices. Supplementation of the media with $\alpha$-tocopherol and selenite resulted in physiological cellular $\alpha$-tocopherol and glutathione peroxidase activity levels, respectively, and significantly protected against $H_2O_2$-induced DNA damage.

## MATERIALS AND METHODS

### *Cell culture*

Cell culture and supplementation studies were performed with the following cell lines: murine fibrosarcoma L929 (DSM ACC 2), human hepatocellular carcinoma Hep G2 (ATCC HB 8065), murine T helper type II cells D10N,[21] human monocytic leukemia cells THP-1 (DSM ACC 16), and human umbilical cord vein endothelial cells ECV 304.[22]

L929 cells, ECV 304, and Hep G2 cells were cultured in 175 $cm^2$ tissue culture flasks (Nunc, Wiesbaden, Germany) in RPMI 1640 medium supplemented with 5% fetal calf serum containing undetectable amounts of $\alpha$-tocopherol ($<$10 nM) and 12.4 $\mu$g selenium/l (Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM alanyl-glutamine (Gibco, Eggenstein, Germany). The THP-1 and D10N[21] cell lines were grown in the same medium but with a FCS batch containing 66 nM $\alpha$-tocopherol and 8.7 $\mu$g Se/l, the D10N got an additional supplementation with 50 $\mu$M $\beta$-mercaptoethanol.

The pretreatment with $\alpha$-tocopherol or selenium was performed for 3–4 d by the addition of the substances to the medium in the form of 1000-fold concentrated stock solutions (in water for selenium or ethanol for $\alpha$-tocopherol, respectively) during the logarithmic growth phase of the cells. A selenium concentration of 50 nM was chosen for the supplementation experiments because this resulted in a maximum of GPx activity in cell homogenates as determined by a concentration-finding pilot experiment using selenite concentrations between 5 and 100 nM.

Adherent cells were harvested by treatment with 12 units dispase® (Boehringer, Mannheim, Germany) in 10 ml saline for 5 min at 37°C (L929) or with 0.125 g trypsin (Hep G2 and ECV 304) in 5 ml water for 4 min at 37°C after washing the cells with PBS. Trypsin activity then was terminated by the addition of serum-containing medium. Suspension cells (D10N) were harvested by centrifugation. Counting was performed in the presence of trypan blue.

### *Characterization of cellular antioxidants*

Harvested cells were washed in PBS and centrifuged at 150 $\times$ $g$ for 8 min. For enzymatic determinations pellets containing 4 $\times$ $10^7$ cells were homogenized in 1.5 ml Tris buffer (100 mM Tris; 300 mM KCl, pH 7.6) containing 0.1% peroxide-free Triton X-100 (Boehringer, Mannheim, Germany) by sonification on a Sonoplus HD70 (Bandelin electronics, Berlin, Germany) with 10 strokes at 70% energy and 30% duty cycle. Determinations of GPx activity were performed with a coupled assay using glutathione reductase to keep the glutathione concentration (3 mM) constant as described, using 80 $\mu$M $H_2O_2$ as substrate for cGPx,[23,24] and 50 $\mu$M phosphatidylcholine hydroperoxide as substrate for PHGPx.[25,26] For the analysis of $\alpha$-tocopherol content the cell homogenate (from 20 $\times$ $10^6$ cells in 1 ml) was mixed with 1 ml EtOH and then extracted three times with 2 ml hexane. $\alpha$-Tocopherol was quantitated by HPLC analysis as described.[27] Selenium was determined in the lyophilized pellet of 4.5 $\times$ $10^7$ cells by neutron activation analysis[28] and in sera by atomic absorption spectrometry,[29] respectively. Total glutathione content was determined according to Tietze[30] after lysis and extraction of cells within the culture plate by 10 mM HCl, 1 mM EDTA. Protein was determined with Coomassie reagent (Biorad, Munich, Germany) according to ref. 31. Growth was optimal at a fetal calf

serum concentration of 5% and was not significantly modified by the addition of selenite.

*Determinations of $H_2O_2$-induced Cytotoxicity.* For cytotoxicity assays L929 cells grown in medium supplemented with 30 $\mu$M $\alpha$-tocopherol, 50 nM sodium selenite or without any special supplement were seeded in 96-well microtiter plates at a concentration of $3 \times 10^5$ ml in a volume of 200 $\mu$l RPMI 1640 containing 5% serum and $\alpha$-tocopherol, selenium, or no special supplement, respectively. Cells were left to adhere overnight before the medium was exchanged for 150 $\mu$l RPMI 1640 without serum then subjected to serial dilutions of $H_2O_2$ in RPMI 1640 and incubated for times indicated in Fig. 5. The reduced capacity of cells to form formazan from MTT (1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)[32] was then determined as a measure for viability. Briefly, 20 $\mu$l 5 mg/ml MTT were added to the cultures and incubation was continued for 30 min at 37°C before supernatants were removed and cells were lysed in isopropanol/formic acid. Viability was defined as the percentage of absorbance of treated cells in comparison to untreated control cells incubated for the same time. Control absorbances ranged between 0.4 and 0.5 absorbance units. Concentration–response curves were calculated by nonlinear regression fitted by the least-square approach to a sigmoid form. Confidence intervals (95%) were calculated with the inplot 4.0 software (Graphpad Inc., Sorrento Valley, CA). The results of the MTT-assay were controlled by parallel measurement of lactate dehydrogenase-release,[33] which yielded the same results.

*Comet Assay for DNA Single Strand Breaks.* The comet assay was performed with L929 cells essentially as described.[18,19] Briefly, $1.2 \times 10^5$ cells were incubated for 30 min at 37°C in 1 ml serum-free RPMI 1640 containing 0, 15, or 30 $\mu$M $H_2O_2$. After the treatment the incubation mixture was centrifuged at $400 \times g$ for 15 min and the cells were resuspended in 75 $\mu$l of 0.7% liquid low melting point agarose (Sigma Type VII) and immediately immobilized on microscopic slides at 4°C. An aliquot of cells was treated in parallel with $H_2O_2$ for 30 min for subsequent determination of viability, which in this case was tested with the trypan blue exclusion procedure. The embedded cells were lysed in situ at 4°C for 60 min (with 2.5 M NaCl, 100 mM EDTA, 10% DMSO, 1% Triton X-100, 1% sodium sarcosinate, 10 mM Tris, pH 10). After positioning of the slides into a horizontal electrophoresis chamber (Biometra, Göttingen, Germany) DNA was allowed to unwind and to equilibrate to the electrophoresis buffer (1 mM $Na_2$-EDTA, 300 mM NaOH) for 20 min. Electrophoresis was performed in an ice bath for 25 min at 25 V and 300 mA. Subsequently, slides were neutralized in 400 mM Tris, pH 7.5 and stained with ethidium bromide. Observations of cells were made at 400× magnification by means of fluorescence microscopy (Leica, Wetzlar, Germany) equipped with an excitation filter BP 515–560 and a barrier filter of 580 nm. DNA damage was quantitated by interactive image analysis (Perceptive Instruments, Halstead, UK). A high-resolution CCD video camera was attached to the microscope and images of the cells were displayed on a VGA screen. DNA from cells with undamaged nuclei appeared as a bright circular area, those with single-strand breaks migrated to the anode during electrophoresis and, thus, formed a comet-like structure with a head and a tail, whose length and/or intensity correlated to the DNA damage. The comet assay application software provides an interactive means of measuring and calculating different parameters for image processing. It defines head and tail regions, determines their length, and measures the intensity values for all points within the comet region. In our test system quantification of the percentage of DNA in the tail proved to be the most accurate parameter to judge DNA damage. It is defined as the sum of all the intensity values in the tail region and expressed as the percentage of the total comet intensity. For each data point images of 200 randomly selected cells (100 from each replicate slide) were analyzed.

## RESULTS

### *$\alpha$-Tocopherol and selenium content of commercially available bovine sera*

During optimization experiments for the measurement of $\alpha$-tocopherol or selenium in human plasma we observed that the fetal calf serum routinely used in our laboratory had very low concentrations of these biofactors (Fig. 1). The $\alpha$-tocopherol concentrations ranged from undetectable values (<10 nM) up to 12 $\mu$M. By using 5% fetal calf serum in the cell culture experiments the available concentrations would range from almost zero to 600 nM. The comparison of a series of serum batches from different suppliers (Fig. 1A) revealed, that the $\alpha$-tocopherol content was consistently lower than that of human serum, which usually ranges between 20 and 30 $\mu$M.[27] Some of the 10 different sera tested (Fig. 1A) even had $\alpha$-tocopherol contents of less than 10 nM, which was our detection limit. The selenium content of fetal calf sera (Fig. 1B) ranged between 67 and 450 nM (5.3–34.9 $\mu$g/l) which is significantly lower than that found in adult man (ca. 1 $\mu$M or 80 $\mu$g/l).[34,35] By using 5% fetal calf serum in the cell culture media a numerical concentration of 3.4 to 22 nM would be reached, however, not telling us anything about the form and the availability of the selenium in these sera.

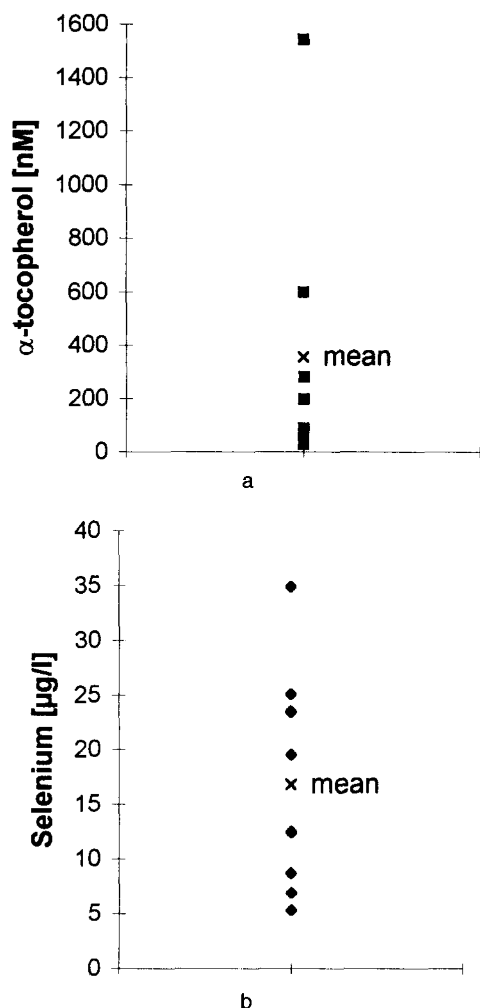


Fig. 1. α-Tocopherol (A) and Selenium (B) content of commercially available sera. Levels ranged and from below detection limit (<10 nM) to 12 μM for α-tocopherol and from 5.3 to 34.9 μg/l for selenium, respectively. α-Tocopherol was estimated by HPLC analysis after hexane extraction.[27] Selenium was measured by means of atomic absorption spectrometry.[29] Individual values (●) shown represent means of three measurements of individual serum batches with the mean thereof (x) indicated.

A general agreement on what could be considered normal serum levels of antioxidants in laboratory animals does not exist. Based on the levels of α-tocopherol and selenium generally accepted as normal in humans, we reasoned that cells grown in standard commercial media without addition of selenite or α-tocopherol and with a supplementation of only 2–10% fetal calf serum should be severely deficient in those micronutrients and that a supplementation of the cell culture medium should increase the cellular antioxidant capacity.

### *α-Tocopherol and selenium content of cultured cells*

In fact, we found in L929 cells that α-tocopherol (30 μM) or selenite (50 nM) supplementation increased cellular contents of α-tocopherol from less than 20 pmol/mg cell protein to 12.8 nmol/mg protein or cellular selenium content from 170 pg/$10^6$ cells (0.7 ng/mg protein) to 617 pg/$10^6$ cells (2.4 ng/mg protein), respectively (Fig. 2). Because the antioxidant effect of selenite is due to its incorporation into glutathione peroxidases, we measured whether the cellular activity of these enzymes was increased. As shown in Figure 3, cGPx activity was increased sevenfold by the addition of sodium selenite to the culture medium, and PHGPx was induced from hardly detectable levels to an activity up to almost 10 mU/$10^7$ cells. Culturing of cells with α-tocopherol did not influence GPx activities (Fig. 3). From these findings we conclude that selenium availability was a limiting factor and that selenite addition enabled the cells to synthesize more potentially cytoprotective enzymes.

To test whether the obvious deficiency in antioxidant defense systems is peculiar for L929 cells or a general phenomenon of cells cultured in conventional tissue culture media, we further investigated the most relevant effect, for instance, the response of glutathione peroxidase activities to selenite supplementation in four other cell lines. As is obvious from Table 1 all cell lines investigated proved to be deficient when cultured in conventional media and comparably responded to selenite supplementation with increase of activities of cGPx and PHGPx. Because only one concentration of selenite (50 nM) was used in these experiments, a differential response of the individual glutathione peroxidases could not be observed.

### *Effect of supplementation on $H_2O_2$-induced genotoxicity*

Next we examined the observations described above for biological relevance, for instance, whether selenium

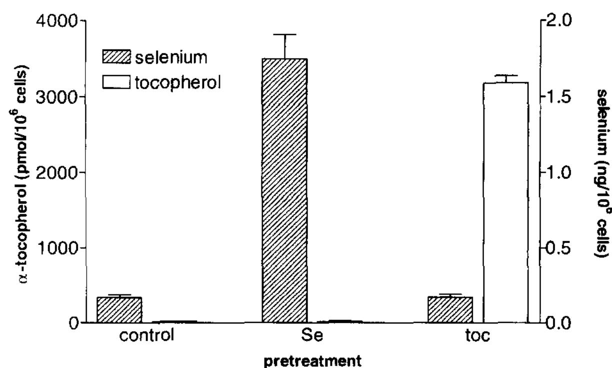


Fig. 2. Selenium and α-tocopherol content of L-929 cells. Cells were cultured for 4 d in control medium as described in Materials and Methods (control), medium containing 30 μM α-tocopherol (toc), or in medium supplemented with 50 nM sodium selenite (Se). Data are means from duplicate (Se) or triplicate (toc) determinations. Analyses were performed as described in Materials and Methods.

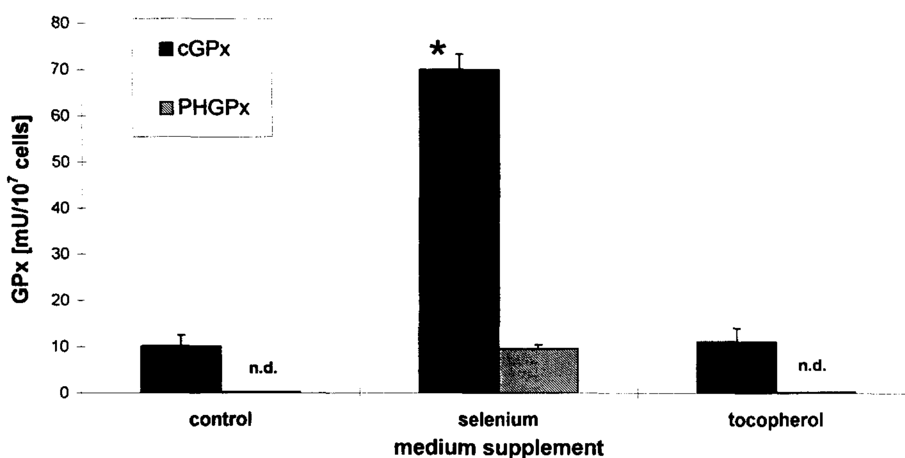


Fig. 3. Activity of classical cytosolic glutathione peroxidase (cGPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) in L-929 cells. Cells were cultured for 4 d in control medium (control), medium containing 30 $\mu$M $\alpha$-tocopherol (tocopherol) or in medium supplemented with 50 nM sodium selenite (selenium). cGPx activity was measured using $H_2O_2$ as substrate, PHGPx was measured with phosphatidylcholin-hydroperoxide (PCOOH) as substrate. Data are means $\pm$ SD of quadruplicate determinations; n.d. means not detectable, $*p < 0.01$. For further details see Materials and Methods.

treatment would make L929 cells more resistant towards hydroperoxide-induced damage. Because DNA is one of the most sensitive cellular targets of $H_2O_2$,[36] we measured whether the induction of single strand breaks due to $H_2O_2$ treatment would be reduced in cells grown in selenite-supplemented medium. DNA single strand breaks may be considered as an early event preceeding cellular transformation or lethality; however, not necessarily leading to a permanent alteration because they can be repaired. We, therefore, chose this parameter as a first read-out system. For the detection of DNA damage on the single cell level we used the comet assay (single cell microgel electrophoresis). We used a hydroperoxide concentration that would induce maximal DNA damage in cells grown in control medium (30 $\mu$M) and a $H_2O_2$ concentration (15 $\mu$M) that induced a strong but submaximal damage to DNA and definitely no cytotoxicity within the treatment period (Fig. 4). Cells precultivated in selenite-enriched medium were virtually completely insensitive towards either concentration of hydroperoxide. Cells grown in $\alpha$-tocopherol–enriched medium were significantly protected only from low concentrations of $H_2O_2$. The observed protection was not due to an altered intracellular glutathione concentration, which was 15.9 $\pm$ 1.6 nmol/mg protein in control cells and did not change significantly in selenite (16 $\pm$ 1.6 nmol/mg protein) or $\alpha$-tocopherol–supplemented (18.9 $\pm$ 2.0 nmol/mg protein) cells.

### *Effect of supplementation on* $H_2O_2$*-induced cytotoxicity*

A further endpoint for demonstration of the protective effect of cellular antioxidant concentration is lethality induced by oxidative stress. The pronounced genotoxic effect of $H_2O_2$ particularly in the selenium deficient cells (Fig. 4) was not paralleled by a loss of viability detectable by the trypan blue exclusion test. $H_2O_2$ (15 $\mu$M) did not show any impairment of viability, and only 0–18% trypan blue stained cells were detected after the 30-min period of 30 $\mu$M $H_2O_2$ treatment. However, because $H_2O_2$-induced cellular damage might result in a delayed loss of viability the long-term effects of $H_2O_2$ exposure on L929, cell viability were investigated systematically by means of their MTT-reducing capacity (Fig. 5). The results obtained with the MTT assay were confirmed by measuring the release of lactate dehydrogenase (not shown). Treatment of cell populations with $H_2O_2$ for short periods (30–120 min), as in the comet assay, proved not to be sufficient to kill all cells even at concentrations of up to 2 mM. We, therefore, challenged cells for 30 or 60 min with increasing concentrations of $H_2O_2$ and then continued the incubation for 9.5 or 9 h, respectively, without $H_2O_2$. A 30-min $H_2O_2$ challenge with postincubation for 9.5 h led to similar results as a simple incubation for 30 min (20–30% dead cells) (not shown). In contrast, a 60 min $H_2O_2$ challenge caused a similarly moderate cell death, whereas a 60-min $H_2O_2$ challenge with 9 h postincubation caused more than 90% cell death at 2 mM $H_2O_2$ (Fig. 5). A similar extent of cell death was obtained, when cells were continuously exposed to $H_2O_2$ for 7 h (data not shown) or 10 h. Both types of toxicity, for instance, the rapid effect seen after 60 min and the more slowly developing toxicity after 10 h was significantly reduced in selenite-pretreated cells, but not significantly altered in $\alpha$-tocopherol–loaded cells. The median lethal concen-

Table 1. Glutathione Peroxidase Activities [mU/$10^7$ Cells] of Cell Lines Cultured with and without Selenium Supplementation

| Cell line | Activity [mU/$10^7$ Cells] Measured with $H_2O_2$ | | Activity [mU/$10^7$ Cells] Measured with PCOOH | |
|---|---|---|---|---|
| | −Se | +Se | −Se | +Se |
| D10N | 26.8 ± 11.6 (7) | 135 ± 6 (2)** | n.d. − 2.2 (9) | 3.3 ± 0.9 (3) |
| ECV-304 | 18.1 ± 6.16 (4) | 280.5 ± 71.9 (6)* | 3.9 ± 2.17 (5) | 38.4 ± 9.77 (5)** |
| Hep G2 | 27.9 ± 4.5 (7) | 284.1 ± 10.3 (2)** | n.d. − 4.0 (5) | 18.1 ± 0.85 (3) |
| THP-1 | 147.2 ± 18.25 (4) | 478.2 ± 93.7 (3)* | 5.3 ± 2.6 (3) | 20.0 ± 0.39 (4)** |

Cells were cultured in the media described in Materials and Methods. The serum used for ECV 304 and Hep G2 cells contained 12.4 ± 0.8 μg selenium/l resulting in a concentration of 7.8 nM in the culture medium, whereas the serum used for D10N and THP-1 cells contained 8.7 ± 0.5 μg selenium/l resulting in 5.5 nM selenium in the culture medium without any information on the bioavailability. In supplementation experiments the cells were freshly seeded in the respective medium containing 50 nM sodium selenite and grown for 3 d. Then cells were harvested and homogenized as described in Materials and Methods and both glutathione peroxidase activites were estimated in the PHGPx buffer system. The activity measured with $H_2O_2$ as substrate is supposed to mainly represent cGPx activities, with PCOOH as substrate only PHGPx should be measured. Values are means ± SD with the number of experiments in parentheses. Statistics were performed by the Student's *t*-test.

*$p < .0005$; **$p < .0001$; n.d. means activities below the detection limit. With series containing zero values statistics analyses were not performed.

tration of $H_2O_2$ (10 h) in cells grown in selenium-supplemented medium was 412 μM (95% confidence interval: 370–457 μM) vs. 172 μM (95% confidence interval: 156–190 μM) in α-tocopherol–loaded cells or 135 μM (95% confidence interval: 110–166 μM) in control cells. Thus, cells grown in selenite-supplemented medium and, therefore, with increased GPx activity (Fig. 3) were three times less sensitive towards $H_2O_2$ toxicity, whereas an increased α-tocopherol content did not influence the outcome of this toxicity experiment using $H_2O_2$ concentrations more than 100-fold higher than those used to initiate DNA single strand breaks. Not surprisingly, however, the protective effect of selenium supplementation is no longer observed beyond a certain threshold of $H_2O_2$ concentrations, which is probably determined by exhaustion of the GSH regenerating system feeding the GSH peroxidases.

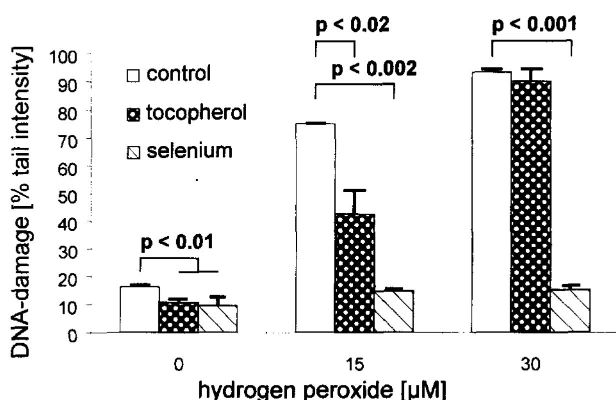


Fig. 4. Protection from DNA strand breaks by α-tocopherol or selenium. Cells were cultured in control medium (control), medium containing 30 μM α-tocopherol (tocopherol) or in medium supplemented with 50 nM sodium selenite (selenium). DNA strand breaks were induced by incubation of intact cells with 15 μM or 30 μM $H_2O_2$ for 30 min and were analyzed in the comet assay. Data are means ± SD from 200 cells. For experimental details see Materials and Methods.

## DISCUSSION

Fetal calf serum is believed to adequately supply cells grown in culture with essential growth factors, vitamins, and trace elements. Because we intended to study the involvement of antioxidants in the cellular response on peroxide challenge, we tried to find sera with particular low contents of α-tocopherol and selenium to grow cells deficient in these antioxidants. Surprisingly, it turned out that most of the commercially available sera *were* deficient in α-tocopherol and selenium, and, as shown in the tests for susceptibility of L929 cells to oxidative stress, this deficiency clearly impairs genetic stability and viability. In this respect, the selenium deficiency appeared to be more relevant than the inadequate α-tocopherol content, although those two micronutrients are generally believed to synergistically prevent oxidative damage.[12] In our study, however, $H_2O_2$, for instance, a direct substrate of GPx, was used as an oxidative challenge and, thus, it is by no means surprising that resaturation of GPx activity proved to be most efficient. In our experimental setting, α-tocopherol supposed to scavenge primarily hydroperoxy radicals of lipids would only interfere with free radical chains initiated by $H_2O_2$ due to inefficient GPx activity.

A systematic investigation of various commercially available sera and batches of sera from the same supplier revealed that in none of them the α-tocopherol and selenium content could be uncritically considered as adequate. The relative selenium deficiency as indicated by reduced activities of GPx that were normalized to a saturating activity by biosynthesis only upon

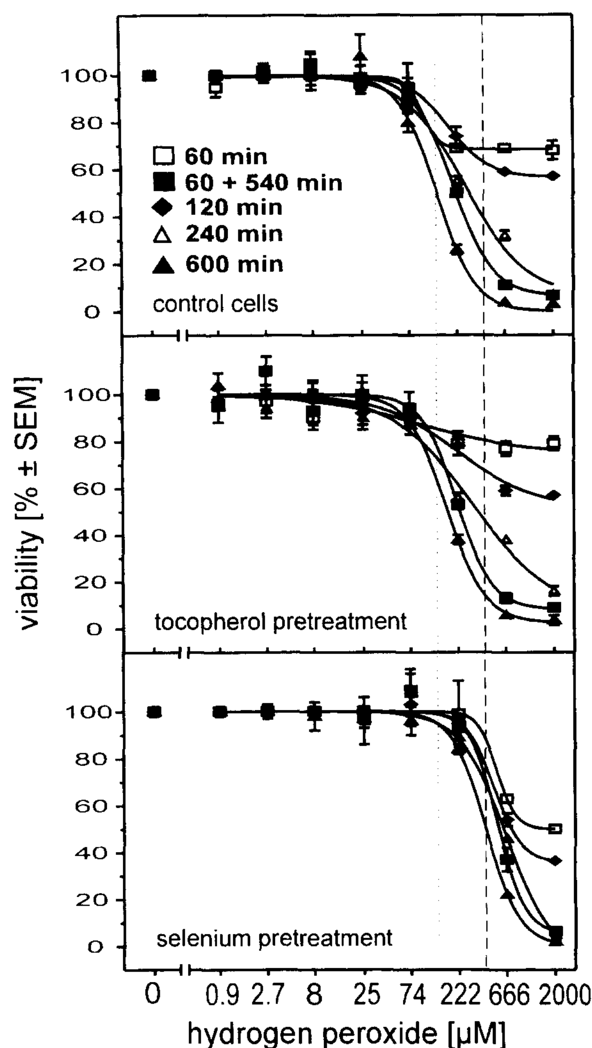


Fig. 5. $H_2O_2$-induced cytotoxicity in L-929 cells. Cells were precultured in control medium, medium containing 30 $\mu$M $\alpha$-tocopherol or in medium supplemented with 50 nM sodium selenite. Then all cells received medium without supplements and were incubated for different time periods up to 600 min with $H_2O_2$ before the influence on viability was determined by measurement of MTT reduction. In addition, cells were incubated 1 h with $H_2O_2$, before the medium was exchanged and the incubation continued for 9 h without $H_2O_2$ (60 + 540 min). The dotted line indicates the median toxic concentration ($TC_{50}$) in the 10 h incubation for control cells. The dashed line indicates the $TC_{50}$ in the 10 h incubation for selenite pretreated cells. Data are means from quadruplicate determinations. For further experimental details see Materials and Methods.

selenite supplementation, was not a peculiarity of L929 cells. Such different cell line as D10N, ECV 304, HepG2, and THP-1, all exhibited marginal, in the case of PHGPx sometimes undetectable, activities of glutathione peroxidase when cultured in such media and responded with a tremendous increase of seleno-enzyme activities upon supplementation with only 40 or 50 nM selenite. Interestingly, the monocytic cell line THP-1 appears to utilize best serum-bound selenium (R.B.F., unpublished data) and also showed highest basal GPx activity (Table 1). It may, therefore, be speculated that the utilization of selenium integrated into proteins of the culture medium depends on the ability of cells to phagocytose or to release proteases.

In fact, a possible interference of proteases used during cell harvesting with our experimental data might be discussed, because (a) residual protease activity might degrade GPx, or (b) it could contribute to selenium utilization during cell propagation and thus increase GPx activity. However, any substantial contributions to our data of trypsin (or dispase®) possibly not completely inhibited by trypsin inhibitor-containing serum addition or by several washing steps, respectively, can be rated as highly unlikely. A set of Hep G2 cells harvested either by trypsination, dispase® treatment, or mechanically yielded identical GPx activities (not shown) and the utilization of serum-bound selenium was poor, irrespective of whether cells had to be exposed to trypsin during propagation for cell detachment or not. The only exception were THP-1 cells, which utilized serum-bound selenium but as suspension cells had never seen trypsin (R.B.F., unpublished observation). Similarly, trypsin had no effect on the $\alpha$-tocopherol content of Hep G2 cells.

Genetic instability is considered a general problem if cell culture systems are to be used in industrial bioprocesses, for example, for the consistent production of recombinant proteins. In this context the significantly deficient vs. supplemented cells (Fig. 4) should raise concern.

Variations in cellular tocopherol and GPx levels might also help to explain discrepancies found in the literature such as the different sensitivity of cells cultured at different serum concentrations[37] towards toxicity caused by the ROS-generator TNF[38] or the different modulation of TNF-toxicity to L-929 cells observed in different laboratories.[39,40] The importance of ROS modulation in cell culture experiments is deduced from the fact that generation of ROS or alteration of antioxidant defense may modulate cellular signalling,[41,42] cell growth,[43] the rate of DNA damage,[44,45] of mutation,[46] tumor initiation, tumor promotion,[2] and the extent or mode of cell death.[43,47–48] Prevention of apoptotic cell death by the putatively antioxidant proteins bcl-2[49,50] or GPx[50] or superoxide dismutase[50] or catalase[51] is not only witnessed in obvious situations of oxidative stress, but also in different physiological models like growth factor withdrawal. A phenomenon that is pathologically often worse than cell death is the survival of DNA-damaged cells and their possible transformation. Such phenomena may occur especially after exposure of cells to oxidants like $H_2O_2$, which induces DNA damage at considerably lower concen-

trations than cell death (this study,[36,52–55]). Because DNA single-strand breaks, which are the predominant form of DNA damage after exposure to $H_2O_2$ are repaired extremely fast,[56] this form of genetic damage does not correlate with cell death, but may still cause some point mutations in the genome. In this context our data, showing that cells with increased GPx or $\alpha$-tocopherol are especially resistant against DNA damage caused by low, noncytotoxic $H_2O_2$ concentrations, suggest that the endogenous antioxidant defense may play an important role in cellular mutagenesis experiments.

The main point of this study was to provide some evidence that very small alterations of cell culture conditions may lead to highly significant changes of the behavior of the whole cell population. For example, the increase of the Se concentration by 50 nM corresponds to only 5% of the concentrations found in human plasma. In our cell culture system, however, such a supplement caused an about fourfold increase in selenium and a sevenfold increase in GPx- activity. These biochemical alterations had a profound influence on the behavior of the whole cell population, because cells were rendered resistant to an otherwise DNA-damaging and toxic stimulus. The absolute values of the cellular selenium concentrations (Fig. 2) are well in the range described previously for different mammalian tissues. In apes, a liver content of 600 ppb and a content in other tissues of 200 ppb was described,[57] which corresponds approximately to 3 ng/mg or 1 ng/mg protein, respectively. In human tissue concentrations of 200–700 ppb were reported (about 1–3 ng Se/mg protein).[58] These data support our assumption that our regulation of Se-consent between 0.7 and 2.4 ng/mg protein may be in the physiological range.

The $\alpha$-tocopherol supplement used corresponds approximately to that found in human plasma.[27,59] The final cellular concentration of $\alpha$-tocopherol approaches that found in erythrocytes (240 $\mu$g/dl)[60] and is in accordance with a study demonstrating the protection of endothelial cells from peroxide toxicity.[61] The extremely low $\alpha$-tocopherol content of bovine sera as compared to that of human serum is neither explained by heat inactivation nor inadequate storage or sample preparation. We also checked whether $\alpha$-tocopherol is substituted by other tocopherols in bovine serum, but found no evidence for this (data not shown). A reduction by up to 80% may be explained from the fact that also in humans there is a maternal to fetal tocopherol ratio of 3:5.[60] The extremely low concentrations found in this study, which led to a virtually complete $\alpha$-tocopherol depletion of cultured cells, however, may rather be explained by tocopherol losses during the preparation procedure of the commercial serum batches.

In conclusion, it is suggested that during the design and interpretation of experiments using cultured cell lines this severe $\alpha$-tocopherol depletion, and the inadequate selenium supply and, therefore, reduced cellular GPx activities should be considered. This antioxidant depletion may not only cause results not corresponding to in vivo situations but may also cause considerable interlaboratory variations of results due to a large variation of $\alpha$-tocopherol or selenium (more than 20-fold) among serum batches.

A standardized supplementation with selenite and $\alpha$-tocopherol seems to be the only practicable way to yield reproducible results in investigations related to oxygen-dependent toxicity and redox modulated signalling and even in industrial tissue culture whenever genetic stability is mandatory.

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