Free Radical Research, 2002 Vol. 36 (9), pp. 957–966

Diethylstilbestrol Attenuates Antioxidant Activities in Testis from Male Mice

TAKAHITO KONDO[a,*], SHINJI GOTO[a], YOSHITO IHARA[a], YOSHISHIGE URATA[a], SATOSHI IKEDA[a], YOSHITAKA HISHIKAWA[b], SHINICHI IZUMI[b], MASASHI SHIN[b] and TAKEHIKO KOJI[b]

[a]*Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan;* [b]*Department of Histology and Cell Biology, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan*

Accepted by Professor E. Niki

(Received 22 October 2001; In revised form 11 March 2002)

It has been reported that acute exposure to diethylstilbestrol (DES) induces apoptosis in the testis, and antioxidants play a role in preventing DES-induced tissue damage. In this study, the effect of chronic exposure to DES on the antioxidants was examined in the testis and liver. Eight-week old male ICR mice were treated subcutaneously with various doses of DES for 20 days. Morphologically apparent apoptotic changes, 4-hydroxy-2-nonenal-positive cells and TUNEL-positive DNA-fragmentation, were demonstrated in the testis, but were minimal in the liver. Activities of antioxidants such as glutathione (GSH) peroxidase and GSH *S*-transferase decreased in both the liver and testis. The activity of Mn-superoxide dismutase (SOD) decreased in the liver but increased in the testis. The activity of Cu, Zn-SOD decreased in the liver but was unchanged in the testis. On Western and Northern blots, gamma-glutamylcysteine synthetase (γ-GCS), a rate limiting enzyme of GSH synthesis, was increased in the liver dependent on the dose of DES. However, the expression of γ-GCS was reduced in the testis. Since quinones, metabolites of DES, generate reactive oxygen species, which damage DNA, antioxidants are important to prevent the damage. The data suggest that antioxidant activities are impaired by DES, and the levels of GSH are related to DES-induced apoptosis in the testis.

Keywords: Diethylstilbestrol; Testis; Liver; Antioxidant; Glutathione; Gamma-glutamylcysteine synthetase

INTRODUCTION

Exogenous hormones such as diethylstilbestrol (DES) adversely affect reproduction causing structural abnormalities, infertility and neoplasia in humans and animals.[1] Carcinogenic potential of DES has been demonstrated in vaginal cell adenocarcinoma and endometrial adenocarcinoma in humans exposed *in utero*.[2] Uterine adenocarcinoma was found in mice treated neonatally with DES.[3] Malignant changes in the murine genital tract closely paralleled those in humans.[4] Maternal exposure to DES in female lambs induces a decrease in testis-size, Sertoli cell numbers, and secretion of follicle-stimulating hormone during fetal life.[5] Prenatal exposure to DES in female mice results in abnormalities of the reproductive tract,[6] which are caused by a suppression of androgen action in response to DES.[7]

DES, a potent synthetic estrogen, interacts with human estrogen receptors.[8] Stimulation of cell proliferation and gene expression through the binding of the estrogen receptor has been implicated as the induction of abnormalities caused by estrogens.[9,10]

Quinone, a genotoxic metabolite of DES, is toxic to many types of organs and cells.[11] Quinone is thought to be needed for DES to bind with DNA and this DNA-DES adduct is believed to be a cause of DNA damage.[12] DES-induced cytotoxicity has been considered to be mediated largely via the generation of reactive oxygen species (ROS).[12–14] The reduction of quinone to hydroquinone and semiquinones by NADPH is catalyzed by flavin-containing enzymes. GSH is important to reduce quinones and prevent the formation of DNA-quinone adducts.[14]

*Corresponding author. Tel.: +81-958-49-7097. Fax: +81-958-49-7100. E-mail: kondo@net.nagasaki-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/107157621000006617

During this redox recycling of quinone/semiquinone, additional ROS, which leads to DNA-damage, is generated.[13,15]

One important function of GSH is the reduction of hydroperoxides through the action of GSH peroxidase (GPX). In order to maintain GSH levels, most cells rely on *de novo* synthesis of GSH via a two-step process catalyzed by γ-GCS and GSH synthetase.[16]

The superoxide generated by quinone/semiquinone recycling is scavenged by Superoxide dismutase (SOD). Glutathione *S*-transferase (GST) catalyzes the formation of GSH adducts with elecrophoretic compounds to efflux outside the cells,[17] and scavenges DNA-damage by oxidative stress such as anti-cancer drugs.[18]

In the testis, prenatal exposure to DES induces cryptochidism. The inhibition of Insulin-like factor 3 mRNA expression by DES may be involved in this process.[5] DES-treatment in adult male rats alters epididymal sperm numbers and sperm parameters, but not sperm production or sperm morphology.[19,20] Acute exposure to DES in rat liver increased the activities of antioxidants such as Cu, Zn-SOD.[21] Many exogenous hormones are speculated to cause oxidative stress in living tissues.[22] An effect of antioxidant on the prevention of quinone-mediated DNA arylation was also reported.[12] However, the role of intracellular antioxidants in DES-induced genotoxicity in testis has not been fully clarified. In this study, we examined the antioxidants in male mice exposed to DES. A possible relationship between apoptotic changes in the testis and antioxidant activities was discussed.

MATERIALS AND METHODS

Animal Experiments

Adult made ICR mice (6–8 weeks; Charles River Japan Inc., Kanagawa, Japan) weighing 30–40 g were used in the study. The mice were injected subcutaneously with various doses of DES dissolved in corn oil or with the vehicle alone every 5 days for total of 20 days. The experimental protocol was approved by the Animal Ethics Committees at our institutions.

Preparation of Tissues

After 20 days exposure, mice were sacrificed under anesthesia. The testis and liver were cut into small pieces either quickly frozen or stocked at −80°C for use in the extraction of protein and RNA, or else fixed in 4% PFA in solution buffer (10 mM sodium phosphate buffer, pH 7.4, 0.137 M NaCl, 0.5 mM EDTA, 0.2 mM PMSF, pepstatin 2 μg/ml, leupeptin 2 μg/ml, PBS) at 4°C overnight and embedded in paraffin using standard procedures. Some sections were later stained with hematoxylin and eosin, and used for the histological evaluation of tissue damage.

Enzyme Activity

Tissue samples were treated for 30 s using a polytron homogenizer (Kinematica AG, Switzerland) at 0°C and centrifuged at 15,000 rpm for 10 min at 0°C. The supernatants were used as materials for the enzymatic analysis. The activity of SOD, GPX and GST was estimated photometrically as described by Beutler *et al.*[23] SOD activity was based on the inhibition of nitroblue tetrazolium reduction by the O_2 produced via photoreduction of riboflavin. A 50% inhibition was defined as 1 unit of SOD activity. The activity not inhibited by potassium cyanide was expressed as Mn-SOD activity. One unit of GPX and GST is expressed as 1 μmol substrate change/min.

Estimation of GSH

After homogenization of the excited tissues in 5 vol. of ice-cold phosphate-buffered saline (9 vol. of 0.154 M NaCl and 1 vol. of 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4) in a Polytron homogenizer, the homogenate treated with trichloroacetic acid was centrifuged at 13,000*g* for 10 min. The supernatant was stored at −80°C until use. The level of GSH was estimated enzymatically as described by Beutler *et al.*[24]

Northern Blots

The probe used was a 764-base pair (bp) DNA fragment (865–1628 bp) of full-length γ-GCS heavy subunit cDNA obtained as described previously.[25] The probe was radiolabeled with [^{32}P]dCTP using Random Primer DNA Labeling kit (Takara Co. Ltd., Tokyo, Japan). Isolation of cytoplasmic RNA and Northern blotting were performed essentially as described by Sambrook *et al.*[26] The cytoplasmic RNA was subjected to electrophoresis in 1% agarose gel containing 0.6 M formaldehyde, subsequently transferred to a nylon membrane, and then hybridized with ^{32}P-labeled probes. Autoradiographed membranes were analyzed using a Fujix Bio-Analyzer BAS-5000 (Fuji Photo Film, Tokyo, Japan). After stripping, the membranes were rehybridized with a ^{32}P-labeled glyceraldehydes 3-phosphate dehydrogenase (GAPDH) probe and the intensity of the bands was estimated. The relative radioactivity was expressed as a ratio of photostimulated luminescence (PSL) corrected by the intensity of GAPDH.

Western Blots

The samples prepared from tissues were preserved at −80°C. The lysate was separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunologically stained using rabbit polyclonal antibody against Cu, Zn-SOD, and Mn-SOD.[27] The bound antibodies were made visible with a horseradish peroxidase coupled secondary antibody using a ECL kit (Amersham Pharmacia Biotech.). The protein concentration was determined according to Redinbaugh and Turley[28] with bovine serum albumin as the standard. The resulting image was saved in the NIH-Image program. Mean density was calculated from a calibration of the Kodak scale using measured "step" values vs. the given "step" values and converting this from the gray scale to optical density units using Rodbard, an equation which takes into account that the conversion is not linear. This equation is part of the NIH-image program. Data was exported to a Stat View (Abacus) spread sheet.

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded tissue blocks from the liver and testis were sectioned at 5 μm and subjected to indirect immunohistochemical staining for 4-hydroxy-2-nonenal (HNE) modified proteins. The sections were dehydrated with toluene and ethanol. After a wash with distilled water, they were immersed in 0.3%. H_2O_2 in methanol for 15 min to block the endogenous peroxidase activity. The sections were then incubated with mouse anti-HNE IgG (0.5 μg/ml) (NOF, Tokyo, Japan) for 1 h.[29] After washing with PBS, the section were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (×100) for 1 h. The HRP sites were visualized in a chromogen solution containing 0.2 mg/ml DAB and 0.005% H_2O_2. They were dehydrated with graded ethanol solutions, cleared with xylene and mounted with permount resin. The specificity of immunohistochemical staining for HNE was confirmed by conducting the negative control staining using normal mouse IgG instead of mouse anti-HNE IgG.

TUNEL Staining

To identify nuclei with DNA strand breaks at the cellular level, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to the method of Gavrieli *et al.* with a slight modification.[30] Briefly, paraffin sections (5–6 μm) were placed onto slain-coated glass slides, dewaxed with toluene and rehydrated with serial ethanol. After being washed with PBS, the sections were treated with 5 μg/ml of proteinase K in PBS at 37°C for 15 min. They were then rinsed once with deionized distilled water (DDW) and incubated with TdT buffer [125 mM Tris/HCl buffer (pH 6.6) containing 0.2 M potassium cacodylate and 0.25 mg/ml BSA] alone at room temperature for 30 min. After the incubation, the slides were reacted with 200 U/ml of TdT dissolved in TdT buffer supplemented with 5 μM biotin-16 μM-dUTP, 20 μM dATP, 1.5 mM $CoCl_2$, and 0.1 mM dithiothreitol at 37°C for 1 h. The reaction was terminated by washing with 50 mM Tris/HCl buffer (pH 7.4) for 15 min. Endogenous peroxidase activity was inhibited by immersing the slides in 0.3%. H_2O_2 in methanol at room temperature for 15 min. The signals were detected immunohistochemically with HRP-conjugated goat anti-biotin antibody, as described previously.[31]

Statistical Analysis

The data are given as the mean ± SD. Differences were calculated with Dunnett's test. Values of $p < 0.05$ were considered significant.

RESULTS

Antioxidants in Liver

Table I shows the effect of DES on antioxidants in the liver. Eight-week old male mice were treated intracutaneously with various concentrations of DES in corn oil for 20 days. As a control, mice were treated with corn oil without DES for 20 days. The level of GSH in the liver increased dependent on the dose of DES and reached 118% at 20 mg/kg of DES (52.1 ± 5.1 nmol/mg protein vs. 44.2 ± 4.5 nmol/mg protein, $p < 0.05$). The level of GSSG, by contrast, did not change. The activities of antioxidants, such as GPX, GST, and SOD, decreased in response to DES. The activity of GPX decreased dependent on dose. The activity at 20 mg/kg of DES was 65.4% of the control (1.38 ± 0.18 units/mg protein vs. 2.11 ± 0.27 units/mg protein, $p < 0.05$). That of GST at this dose was 57.4% of the control (1.17 ± 0.18 units/mg protein vs. 2.04 ± 0.25 units/mg protein, $p < 0.05$). That of Cu, Zn-SOD at this dose was 82.0% of the control (171.8 ± 39.1 vs. 209.6 ± 0.4 milliunits/mg protein, $p < 0.05$) and that of Mn-SOD 45.2% (19.3 ± 1.6 vs. 42.7 ± 4.6 milliunits/mg protein, $p < 0.05$).

Antioxidants in Testis

Table II shows the effect of DES on antioxidants in the testis. The levels of total glutathione (GSH + GSSG) in testis decreased gradually dependent on dose and was 79.3% at 20 mg/kg (48.9 ± 2.5 vs. 66.7 ± 2.0 nmol/mg protein, $p < 0.05$). The levels

TABLE I Antioxidants in liver

Condition	Total GSH (nmol/mg protein)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GPX (units/mg protein)	GST (units/mg protein)	Total SOD (milli-units/mg protein)	Cu, Zn-SOD (milli-units/mg protein)	Mn-SOD (milli-units/mg protein)
Control	44.2 ± 4.5	36.4 ± 3.2	7.8 ± 1.0	2.11 ± 0.27	2.04 ± 0.25	252.3 ± 5.0	209.6 ± 0.4	42.7 ± 4.6
DES (1 mg/kg)	49.6 ± 5.8	41.7 ± 3.5	7.9 ± 1.2	1.80 ± 0.17	1.82 ± 0.43	275.9 ± 11.8	230.9 ± 1.4	45.0 ± 10.4
DES (10 mg/kg)	51.0 ± 2.7*	43.1 ± 3.4*	7.9 ± 1.2	1.51 ± 0.10*	1.28 ± 0.10*	253.9 ± 6.5	226.6 ± 4.3	27.3 ± 2.2
DES (20 mg/kg)	52.1 ± 5.1*	43.9 ± 4.2*	8.2 ± 2.1	1.38 ± 0.18*	1.17 ± 0.18*	191.1 ± 40.6*	171.8 ± 39.1*	19.3 ± 1.6*

Values are the mean ± SD. *$p < 0.05$ vs. control.

TABLE II Antioxidants in testis

Condition	Total GSH (nmol/mg protein)	GPX (units/mg protein)	GST (units/mg protein)	Total SOD (milliunits/mg protein)	Cu, Zn-SOD (milliunits/mg protein)	Mn-SOD (milliunits/mg protein)
Control	66.7 ± 2.0	0.18 ± 0.09	0.42 ± 0.03	161.0 ± 6.6	149.5 ± 5.9	11.6 ± 0.7
DES (1 mg/kg)	67.1 ± 16.8	0.18 ± 0.04	0.32 ± 0.05*	161.3 ± 32.4	139.4 ± 29.7	21.9 ± 2.7*
DES (10 mg/kg)	59.6 ± 0.8	0.13 ± 0.05*	0.29 ± 0.01*	165.9 ± 9.1	150.1 ± 8.2	15.8 ± 0.9
DES (20 mg/kg)	48.9 ± 2.5*	0.10 ± 0.01*	0.24 ± 0.01*	164.8 ± 12.6	148.1 ± 9.6	16.7 ± 3.1*

Values are the mean ± SD. *$p < 0.05$ vs. control.

of GSSG were not determined because of insufficient materials. The activity of GPX decreased in a dose-dependent manner. The GPX activity at 20 mg/kg of DES was 55.6% of the control (0.10 ± 0.01 vs. 0.18 ± 0.09 units/mg protein, $p < 0.05$). That of GST was 57.1% (0.24 ± 0.01 vs. 0.42 ± 0.03 units/mg protein, $p < 0.05$). The total activity of SOD and the activity of Cu, Zn-SOD did not change on treatment with DES. Only, the activity of Mn-SOD increased on treatment with DES, independent of the dose (136–189% of the control).

Expression of Antioxidants

Figure 1A,B shows immunoblots of the γ-GCS heavy subunit (γ-GCSh), a catalytic subunit, in the liver and testis. In the liver, DES-exposure increased the expression of γ-GCSh concomitant with the change in the level of GSH. On the other hand, the expression of γ-GCSh decreased at 20 mg/kg of DES in the testis. Figure 1C,D shows Northern blots of the γ-GCSh. The expression of γ-GCSh mRNA increased in the liver (lanes 1 and 2) but, decreased in the testis (lanes 3 and 4) following exposure to DES. Figure 2 shows immunoblots of Cu, Zn-SOD. The expression of Cu, Zn-SOD decreased in liver, whereas it was not changed in testis. These results were concomitant with the activity of Cu, Zn-SOD in these tissues. Figure 3 shows immunoblots of Mn-SOD. A remarkable decrease in the expression of Mn-SOD was observed in the liver at 20 mg/kg of DES, corresponding to the decrease in activity. The expression of Mn-SOD increased by 1.4-fold at 20 mg/kg in the testis.

TUNEL Staining

The induction of cell death in the liver and testis was assessed by TUNEL staining. Figure 4 shows the results. Essentially, very few TUNEL-positive cells were detected in the sections of liver (upper left) and testis (lower left) from control mice. In the liver, DES (20 mg/kg)-treatment increased the number of TUNEL-positive cells and these cells were regarded as littoral cells, but not parenchymal cells (upper right). In the testis, DES induced an increased

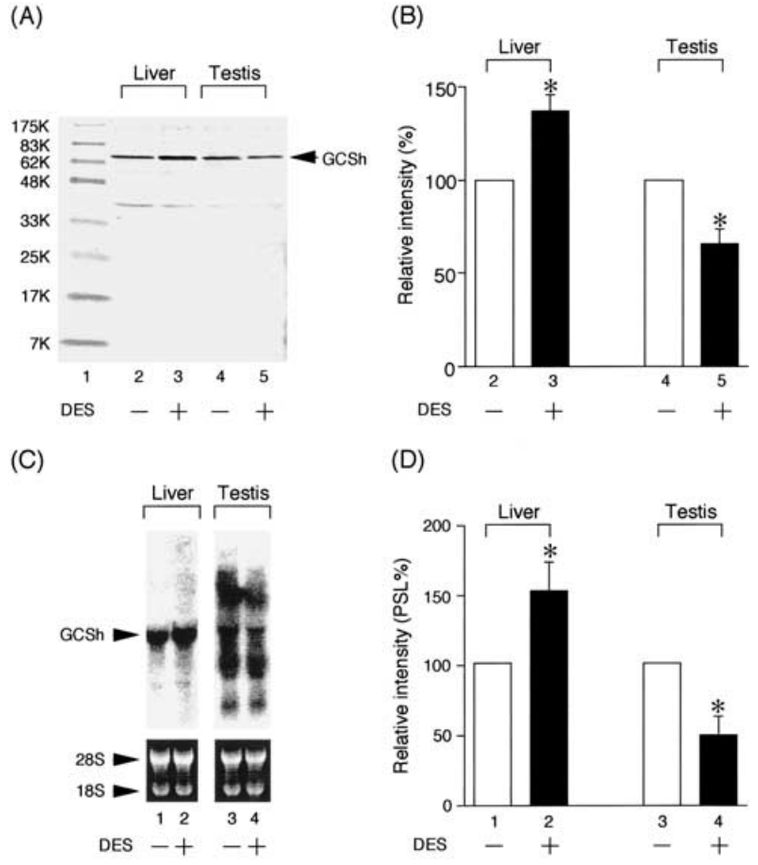

FIGURE 1 Expression of γ-GCS. Levels of γ-GCS heavy subunit protein were estimated by Western blot analysis (A). Relative amounts of immunological activity are expressed as a percentage of the control (B). Each lane corresponds to the one in (A). The expression of γ-GCSh mRNA was estimated by Northern blot analysis (C). Values were normalized to the GAPDH mRNA level and expressed as relative intensity (PSL%) taking the level of γ-GCS in the control as 100% (D). Each lane corresponds to the one in (C). The data are the mean ± SD of three independent analyses. *$p < 0.05$ vs. each control.

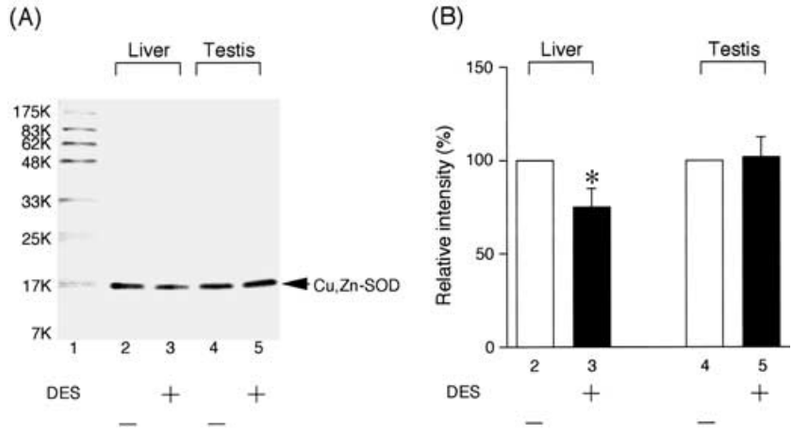

FIGURE 2 Western blots of Cu, Zn-SOD. Levels of Cu, Zn-SOD protein were estimated by Western blot analysis (A). Relative amounts of immunological activity are expressed as a percentage of the control (B). Each lane corresponds to the one in (A). The data are the mean ± SD of three independent analyses. *$p < 0.05$ vs. control.

in TUNEL-positive germ cells, which were mainly spermatogonia and spermatocytes (lower right). The rate of apoptotic cells in DES-treated liver were 2.1-fold of the control liver (1.5 ± 0.2 vs. 0.7 ± 0.2, Mean ± SEM, $n = 3$). On average, at least 2000 cells were counted in randomly selected six fields in each sample. That in DES-treated testis were 3.2-fold of the control testis (0.51 ± 0.04 vs. 0.16 ± 0.01 Mean ± SEM, $n = 3$). On average, we counted the number of apoptotic cells per seminiferous tubule as described previously.[31]

Figure 5 shows immunohistochemical staining of HNE. There was no apparent change in the HNE-positive cells between DES (20 mg/kg)-treated liver (B) and corn oil-treated liver (A). On the other hand, the HNE-positive cells were more in DES (20 mg/kg)-treated testis (D) than in corn oil-treated testis (C). Figure 6 shows mirror sections of TUNEL staining and HNE immunostaining in DES (20 mg/kg)-treated mice. Both in liver and testis, there were found cells with simultaneous positive to HNE and TUNEL. This suggests that HNE-positive cells increased by the treatment with DES cause of apoptosis.

DISCUSSION

In this study, changes in the activities of antioxidants were assessed in liver and testis from male mice treated with DES for 20 days. It was found that: (1) TUNEL-positive cells were spread throughout the testis but limited in numbers in the liver; (2) a decrease in the antioxidant activities such as GST and GPX was observed both in liver and testis; (3) levels of GSH and the expression of γ-GCS increased in liver, whereas, they decreased in testis and (4) a reverse correlation was suggested between the levels of GSH and DES-induced DNA damage. This is the first report that impairment of antioxidant activity relates to DES-induced apoptosis in the testis. Since DES is an exogenous estrogen, the

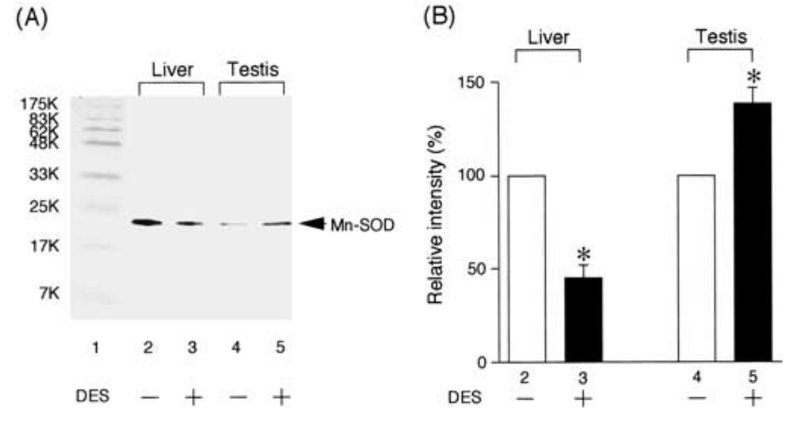

FIGURE 3 Western blots of Mn-SOD. The levels of Mn-SOD protein were estimated by Western blot analysis (A). The relative amounts of the immunological activity are expressed as a percentage of the control (B). Each lane corresponds to the lane in (A). The data are the mean ± SD of three independent analyses. *$p < 0.05$ vs. control.

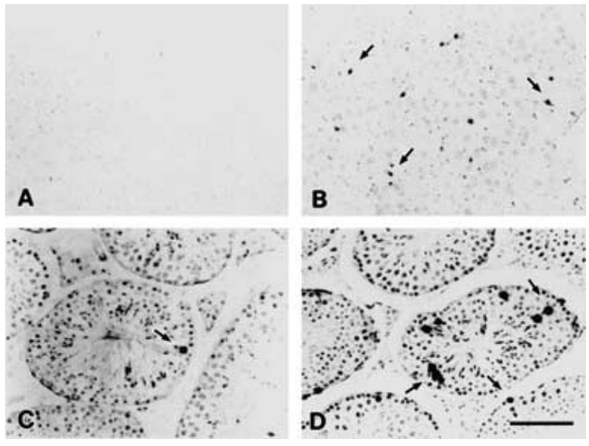

FIGURE 4 TUNEL staining. To identify nuclei with DNA strand breaks at the cellular level, TUNEL staining was performed as described in "Materials and Methods" section. Upper left panel, liver of control; upper right, liver of a DES (20 mg/kg)-treated mouse; lower right, testis of control; lower right; testis of a DES (20 mg/kg)-treated mouse. TUNEL-positive cells appear as black spots.

change in antioxidant activity caused by DES or its metabolites might be due to (1) inhibition of estrogen receptor-mediated signals leading to a change in the gene expression which is regulated by the estrogen receptor; (2) post-translational modification of the antioxidants and (3) utilization of the antioxidants for detoxification.

In the testis, exposure to DES results in apoptotic changes. Apoptosis was reported in germal cells during DES-induced regression of the testis in adult male Syrian hamsters.[32] Apoptosis was found 3 days after exposure to 20 mg/kg of DES, especially in spermatocytes, but cell proliferation was not inhibited. It was also reported that DES-treatment in adult male rats altered epididymal sperm numbers and sperm parameters but not sperm production or sperm morphology.[20] In this study, apoptotic cells observed in DES-exposed liver were regarded as littoral cells, but not parenchymal cells. In DES-exposed testis, apoptotic cells were regarded as spermatogonia and spermatocytes (Fig. 4). Increase

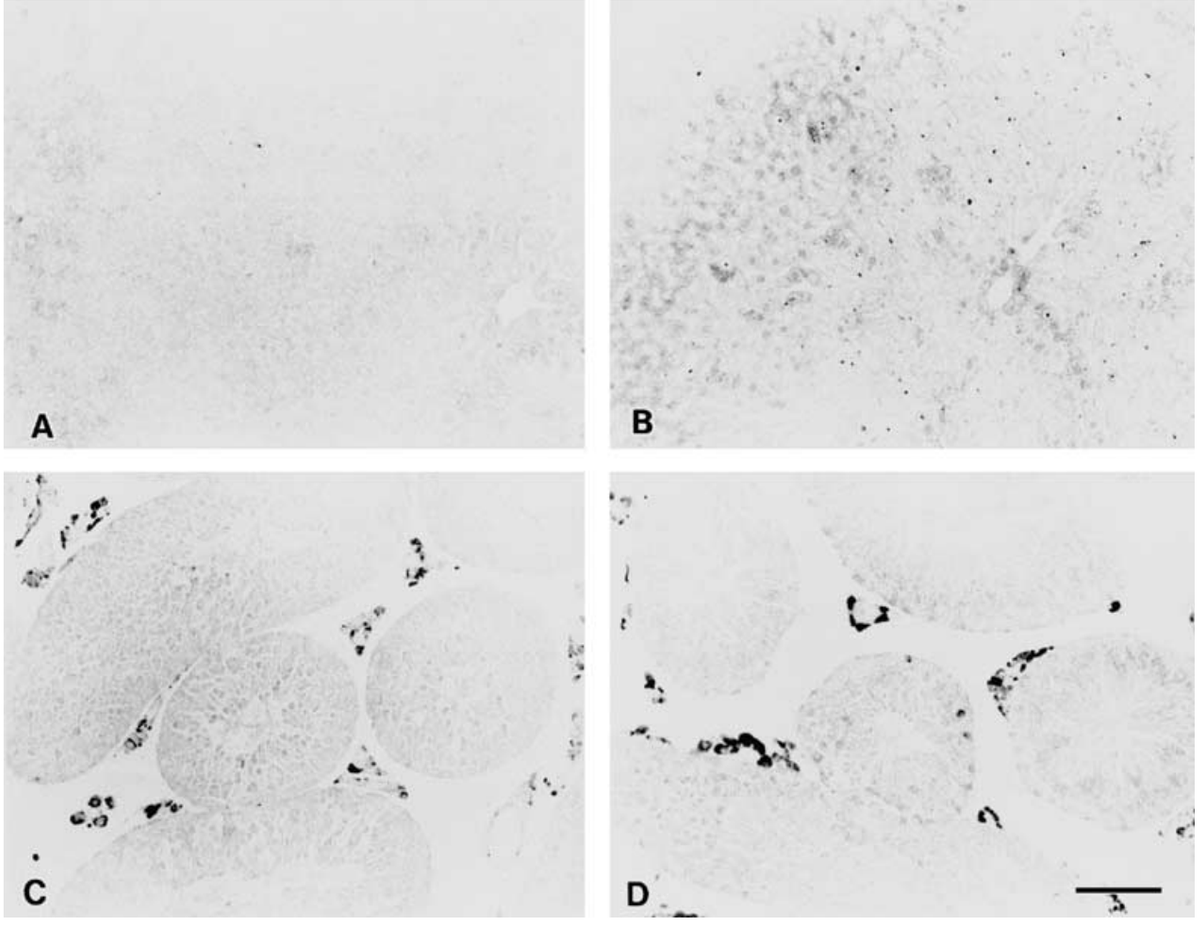

FIGURE 5 Immunohistochemical staining of HNE. Presence of HNE was examined immunohistochemically as describe in "Materials and Methods" section. (A) Corn oil-treated mouse liver, (B) DES (20 mg/kg)-treated mouse liver, (C) corn oil-treated mouse testis, (D) DES (20 mg/kg)-treated mouse testis. HNE-positive cells were shown as black spots. Bars, 50 μm.

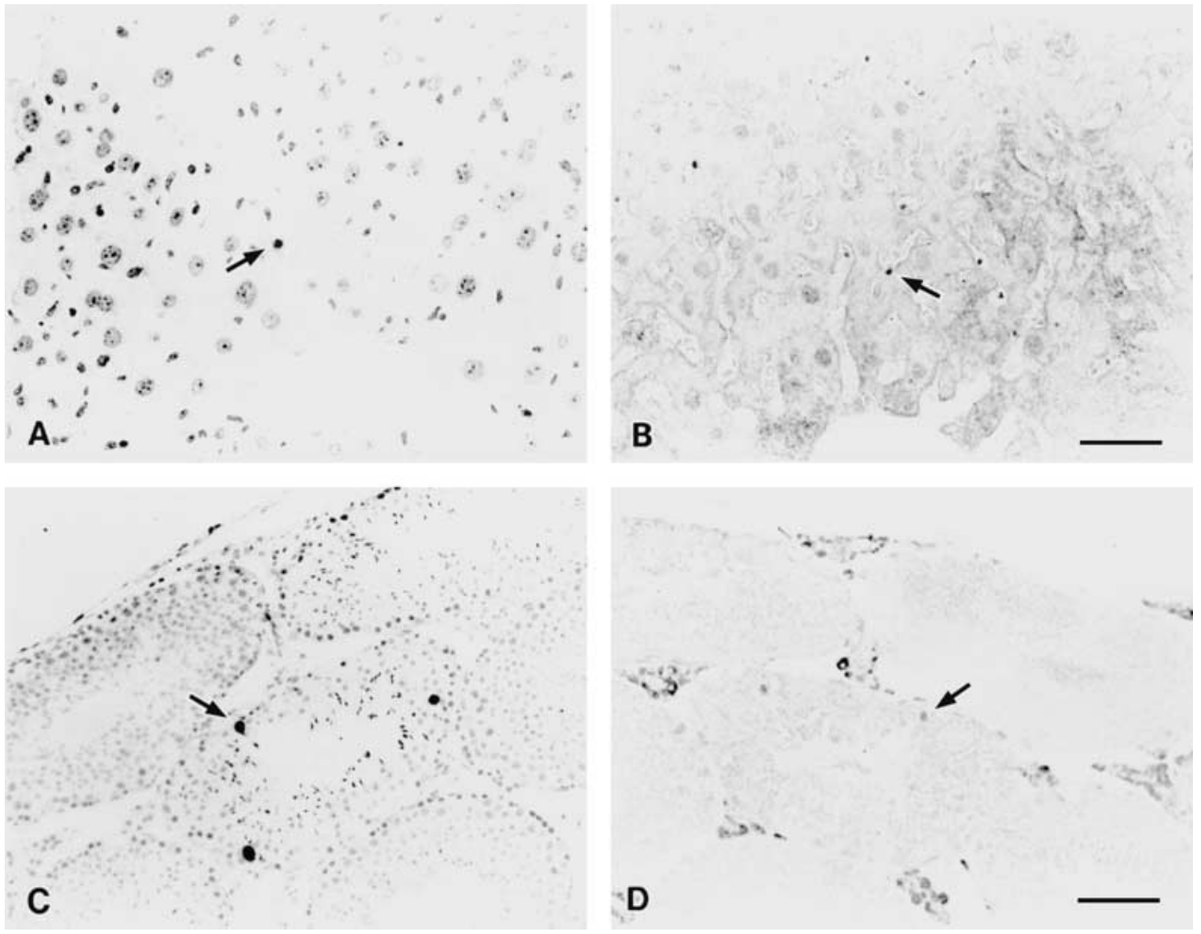

FIGURE 6 Mirror sections of TUNEL staining and HNE immunostaining in DES (20 mg/kg)-treated mice. (A) TUNEL staining of the liver; (B) HNE immunostaining of the liver; (C) TUNEL staining of the testis; (D) HNE immunostaining of the testis. The identical cells were shown as arrows. Bars, 25 μm (A and B), 50 μm (C and D).

in the TUNEL-positive cells by DES were more in the testis than the liver. DES-treatment also increased HNE-positive cells in the testis (Fig. 6), some of which were simultaneously positive to TUNEL (Fig. 4). These results suggest that oxidative stress induced by DES increases lipidperoxidation to lead apoptotic changes in the testis.

In this study, the decrease in the expression of Cu, Zn- and Mn-SOD in the liver of DES-exposed mice was responsive to a decrease in their activities (Figs. 2 and 3). The mechanism for the decrease is unclear. One explanation is that the expression of these enzymes is regulated transcriptionally by estrogens, and DES affects the regulation. On the other hand, both the activity and expression of Mn-SOD increased in the testis of DES-exposed mice. The reason for the difference in the expression of Mn-SOD, located in mitochondria, between liver and testis remains to be clarified, however, it is suggested that change in the expression of Cu, Zn- and Mn-SOD dose not correlate to the damage in DES-exposed mice testis.

The expression of γ-GCS was elevated in the liver at 20 mg/kg of DES (Fig. 1), consistent with the elevation of GSH (Table I). The expression of γ-GCS is regulated in response to oxidative stress, cytokines, growth factors, chemical insults, and anticancer drugs and up-regulated by antioxidant response elements in the promoter region.[33] In the current study, there was no apparent apoptotic change in hepatocytes, suggesting that upregulation of GSH synthesis in the liver is important in preventing oxidative damage by DES (Fig. 4).

In the testis, the activities of GPX and GST decreased on treatment with DES as observed in the liver. GPX is present mainly in the cytosol and has several isozymes in plasma, plasma membrane, and mitochondria. These isozymes scavenge hydrogen peroxide to protect proteins and lipids. GST detoxifies a broad range of electrophilic compounds, and protects against the lipid peroxidation of membranes and DNA-damage. Regarding quinones, NAD(P)H:quinone reductase 1 as well as GST Ya is expressed coordinately with the γ-GCS gene

in rats.[34] This suggests that these antioxidant enzymes are expressed in response to oxidative stress.

The most striking difference between the liver and testis in mice exposed to DES was the change in the level of GSH. GSH participates in many biological processes, including cellular defense against oxidative stress, by reducing the disulfide linkage of proteins and other cellular molecules, or by scavenging ROS. Cells resistant to oxidative stress possess large amounts of GSH and express γ-GCS mRNA in response to these stimuli. Basically, the liver possesses the most GSH of all mammalian tissues and levels were further elevated in response to DES (Table I). This increase may reflect up-regulation of γ-GCS gene expression by DES and a strengthening of resistance to DES-induced damage. Another important function of GSH is to maintain the redox potential within cells. Many intracellular signal pathways are regulated by GSH. Changes in the levels of GSH modulate these signal activities. Intracellular signals regulated by GSH are important for cell survival, proliferation and development.

The levels of GSH in the testis decreased following DES-treatment. It is notable that in control mice, the levels in the testis and liver were the same. The activity of GPX in control testis was 9% of that in liver, that of GST, 21%, and total SOD, 64%. This suggests the importance of GSH in maintaining function in the testis. Decreases in the levels of GSH in the testis of DES-treated mice might increase the sensitivity to DES-induced damage.

There is no evidence to show the direct relationship between decrease in the GSH level and increase in the apoptotic change in the testis. However, there have been many reports on the significance of GSH to prevent apoptosis.[35,36] It is speculated decrease in the GSH synthesis is, at least, involved in the impairment of spermatogonia and spermatocytes by DES. However, difference in the mechanism for DES-induced apoptotic change in liver and testis is not clarified at present.

In this experiment, the expression of γ-GCS was reduced in the testis of DES-exposed mice. It is not clear why the expression was down-regulated in the testis, but up-regulated in the liver. However, it is suggested that the suppression contributes to a decrease in GSH, which weakens the redox cycle of quinone and enhances production of ROS and the formation of DNA-quinone adducts leading to apoptosis.

References

[1] McLachlan, J.A., Newbold, R.R. and Bullock, B.C. (1980) "Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol", *Cancer Res.* **40**, 3988–3999.

[2] Keller, C., Nanda, R., Shannon, R.L., Amit, A. and Kaplan, A.L. (2001) "Concurrent primaries of vaginal clear cell adenocarcinoma endometrial adenocarcinoma in a 39-year old women with in utero diethylstilbestrol exposure", *Int. J. Gynecol. Cancer* **11**, 247–250.

[3] Newbold, R.R., Banks, E.P., Bullock, B. and Jecfferson, W.N. (2001) "Uterine adenocarcinoma in mice treated neonatally with genistein", *Cancer Res.* **61**, 4325–4328.

[4] Newbold, R.R., Bullock, B.C. and McLachlan, J.A. (1990) "Uterine adenocarcinoma in mice following developmental treatment with estrogens: a model for hormonal carcinogenesis", *Cancer Res.* **50**, 7677–7681.

[5] Emmen, J.M., McLuskey, A., Adham, I.M., Engel, W., Verhorf-Post, M., Themmen, A.P., Grootegoed, J.A. and Brinkmann, A.O. (2000) "Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism", *Endocrinology* **141**, 846–849.

[6] Newbold, R.R., Hanson, R.B., Jefferson, W.N., Bullock, B.C., Haseman, J. and McLachlan, J.A. (2000) "Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol", *Carcinogenesis* **21**, 1355–1363.

[7] Nikov, G.N., Eshete, M., Rajnarayanan, R.V. and Alworth, W.L. (2001) "Interactions of synthetic estrogens with human estrogen receptors", *J. Endocrinol.* **170**, 137–145.

[8] Bullock, B.C., Newbold, R.R. and Jeferson, K.S. (1997) "Transgenic mice with aberrant expression of the estrogen receptor demonstrate accelerated onset of uterine tumors after neonatal exposure to diethylstilbestrol", *Mol. Carcinog.* **19**, 236–242.

[9] McKinnell, C., Atanassova, N., Williams, K., Fisher, J.S., Walker, M., Tuner, K.J., Saunders, T.K. and Sharpe, R.M. (2001) "Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol", *J. Androl.* **22**, 323–338.

[10] Couse, J.F., Davis, V.L., Hanson, R.B., Jefferson, W.N., McLachlan, J.A., Bullock, B.C., Neubold, R.R. and Korach, K.S. (1997) "Accelerated onset of uterine tumors in transgenic mice with aberrant expression of the estrogen receptor after neonatal exposure to diethylstilbestrol", *Mol. Carcinog.* **19**, 236–242.

[11] Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) "The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes", *J. Biol. Chem.* **127**, 270–276.

[12] Sharma, M. and Slocum, H.K. (1999) "Prevention of quinone-mediated DNA arylation by antioxidants", *Biochem. Biophys. Res. Commun.* **262**, 769–774.

[13] Shi, M., Gozal, E., Choy, H.A. and Forman, H.J. (1993) "Extracellular glutathione and gamma-glutamyl transpeptidase prevent H_2O_2-induced injury by 2,3-dimethoxy-1,4-naphthoquinone", *Free Radic. Biol. Med.* **15**, 57–67.

[14] Forman, H.J., Shi, M., Kugelman, A., Iwamoto, T. and Choy, H.A. (1995) "γ-Glutamyl cycle enzymes and antioxidant defense", In: Davies, K.J.A. and Ursini, F., eds, The Oxygen Paradox (Cleup University Press, Italy), pp 223–234.

[15] Zhang, L., Bandy, B. and Davison, A.L. (1996) "Effects of metals, ligands and antioxidants on the reaction of oxygen with 1,2,4-benzenetriol", *Free Radic. Biol. Med.* **20**, 495–505.

[16] Meister, A. (1985) "Methods for the selective modification of glutathione metabolism and study of glutathione transport", *Methods Enzymol.* **113**, 571–585.

[17] Chang, M., Shi, M. and Forman, H.J. (1992) "Exogenous glutathione projects endothelial cells from menadione toxicity", *Am. J. Physiol.* **6**, L 637–643.

[18] Goto, S., Ihara, Y., Urata, Y., Izumi, S., Abe, K., Koji, T. and Kondo, T. (2001) "Doxorubicin-induced DNA intercalation and scavenging by the nuclear glutathione *S*-transferase π", *FASEB J.* **15**, 2702–2714.

[19] Atanassova, N., McKinnell, C., Walker, M., Turner, K.J., Fisher, J.S., Morley, M., Millar, M.R., Groome, N.P. and Sharpe, R.M. (1999) "Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, sertoli cell number, and the efficiency of spermatogenesis in adulthood", *Endocrinology* **140**, 5364–5373.

[20] Goyal, H.O., Braden, T.D., Mansour, M., Williams, C.S., Kamaleldin, A. and Srivastava, K.K. (2001) "Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but without alterations in sperm production and sperm morphology", *Biol. Reprod.* **64**, 927–934.

[21] Genc, S., Gurdol, F., Oner-Iyidogan, Y. and Suzme, R. (1999) "Acute effects of estradiol and of diethylstilbestrol: pro- or antioxidant potential?", *Res. Commun. Mol. Pathol. Pharmacol.* **105**, 253–261.

[22] Liehr, J.G., Roy, D. and Gladek, A. (1989) "Mechanism of inhibition of estrogen-induced renal carcinogenesis in male Syrian hamsters by vitamin C", *Carcinogenesis* **10**, 1983–1988.

[23] Beutler, E. (1984) "A manual of biochemical methods", In: Beutler, E., ed, Red Cell Metabolism, 3rd Ed. (Grune and Stratton, Orlando, FL), pp 77–78.

[24] Beutler, E. (1984) "A manual of biochemical methods", In: Beutler, E., ed, Red Cell Metabolism, 3rd Ed. (Grune and Stratton, Orlando, FL), pp 131–136.

[25] Iida, T., Mori, E., Mori, K., Goto, S., Urata, Y., Oka, M., Kohno, S. and Kondo, T. (1999) "Co-expression of gamma-glutamylcysteine synthetase sub-units in response to cisplatin and doxorubicin in human cancer cells", *Int. J. Cancer* **82**, 405–411.

[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) "Extraction, purification, and analysis of messenger RNA from eukaryotic cell", Molecular Cloning: A Laboratory Manual, 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp 7–39.

[27] Suzuki, K., Tatsumi, H., Satoh, S., Senda, T., Nakata, T., Fujii, J. and Taniguchi, N. (1993) "Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction", *Am. J. Physiol.* **265**, H1173–H1178.

[28] Redinbaugh, M.G. and Turley, R.B. (1986) "Adaptation of the bicinchoninic acid protein assay for use with microtiter plates", *Anal. Biochem.* **153**, 267–271.

[29] Ejima, K., Koji, T., Tsuruta, D., Nanri, H., Kashimura, M. and Ikeda, M. (2000) "Induction of apoptosis in placentas of pregnant mice exposed to lipopolysaccharides: possible involvement of Fas/Fas ligand system", *Biol. Reprod.* **62**, 178–185.

[30] Gavrieli, Y., Sherman, Y. and Ben-Sasson, S.A. (1992) "Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation", *J. Cell. Biol.* **119**, 493–501.

[31] Koji, T., Hishikawa, Y., Ando, H., Nakanishi, Y. and Kobayashi, N. (2001) "Expression of Fas and Fas ligand in normal and ischemia-reperfusion testes: involvement of the Fas system in the induction of germ cell apoptosis in the damaged mouse testis", *Biol. Reprod.* **64**, 946–954.

[32] Nonslercq, D., Reverse, D., Toubeau, G., Beckers, J.F., Sulon, J., Laurent, G., Zanen, J. and Heuson-Stiennon, J.A. (1996) "*In situ* demonstration of germinal cell apoptosis during diethylstilbestrol-induced testis regression in adult male Syrian hamsters", *Biol. Reprod.* **55**, 1368–1376.

[33] Kondo, T., Higashiyama, Y., Goto, S., Iida, T., Cho, S., Iwanaga, M., Mori, K., Tani, M. and Urata, Y. (1999) "Regulation of γ-glutamylcysteine synthetase expression in response to oxidative stress", *Free Radic. Res.* **31**, 325–334.

[34] Murphy, T.H., Yu, J., Ng, R., Johnson, D.A., Shen, H., Honey, C.R. and Johnson, J.A. (2001) "Preferential expression of antioxidant response element mediated gene expression in astrocytes", *J. Neurochem.* **76**, 1670–1678.

[35] Kitao, M., Akao, Y., Ohishi, N., Yagi, K. and Nozawa, Y. (2002) "Arsenic trioxide-induced apoptosis and its enhancement by buthionine sulfoximine in hepatocellular carcinoma cell lines", *Biochem. Biophys. Res. Commun.* **291**, 861–867.

[36] Troyano, A., Fernandez, C., Sancho, P., de Blas, E. and Aller, P. (2001) "Effect of glutathione depletion on antitumor drug toxicity (apoptosis and necrosis) in U-937 human promonocytic cells. The role of intracellular oxidation", *J. Biol. Chem.* **276**, 47107–47115.