ELSEVIER

Mutation Research 483 (2001) 27–33

Fundamental and Molecular Mechanisms of Mutagenesis

www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

The inhibition of DNA repair capacity by stilbene estrogen in Leydig cells: its implications in the induction of instability in the testicular genome

James W. DuMond, Deodutta Roy*

Department of Environmental Health Sciences, University of Alabama at Birmingham, 317 Ryals Building, 1665 University Blvd., Birmingham, AL 35294-0022, USA

Received 19 March 2001; received in revised form 21 May 2001; accepted 1 June 2001

Abstract

In this study, we examined the effect of stilbene estrogen, diethylstilbestrol (DES), on the DNA repair capacity of mouse Leydig cells using the host cell reactivation assay. Cells transfected with UV-damaged plasmids, undamaged plasmids, or no plasmids (sham treatment) were grown in serum, serum-free, or DES plus serum-free medium. The serum-grown cells which have a shorter cell cycle time (16 h) showed a 40% decrease in DNA repair capacity when compared to serum-free cells with a longer cell cycle time (25 h). A significant decrease in the DNA repair capacity of the Leydig cells exposed to DES was observed compared to untreated cells grown in a serum-free environment ($P < 0.05$). The effect of DES on DNA repair in Leydig cells was dose dependent. We have recently shown that DES stimulates the growth of Leydig cells. Stimulatory growth of Leydig cells coupled with a decrease in DNA repair capacity by DES may allow the accumulation of mutagenic lesions in DNA. The mutagenic lesions may result from the attack of redox cycling products of DES and/or errors of replication. This, in turn, may produce alterations in the genome of Leydig cells resulting in genetically unstable cells that may serve as precursor cells for testicular carcinogenesis.

Keywords: DNA repair; Host cell reactivation; Leydig cells; DES; Estrogen

1. Introduction

Stilbene estrogen (diethylstilbestrol, DES) is a hormonal chemical that possesses potent estrogenic activity. It is carcinogenic in both animals and humans [1]. In utero exposure of DES has been reported to induce sexual malformations in both humans and animals [2–4]. DES has also been linked to the development of clear cell adenocarcinoma of the vagina in humans [5]. 17β-Estradiol and DES are testicular carcinogens in animals [2,6]. The mechanism by which estrogen might initiate testicular tumorigenic events is not clear. It is widely accepted that genetic instability in the target organ is required for the development of cancer [7]. Mutations in a minimum of three to four candidate target genes in the same progenitor cells are essential for the malignant transformation in vivo [8]. The mechanisms by which estrogens might incorporate genetic alterations in progenitor cells are highly controversial. We previously demonstrated that catecholestrogen and DES participate in the redox cycling reactions catalyzed by microsomal, mitochondrial

* Corresponding author. Tel.: +1-205-934-6081; fax: +1-205-975-6341.
E-mail address: royd@uab.edu (D. Roy).

0027-5107/01/$ – see front matter
PII: S0027-5107(01)00222-6

and nuclear enzymes [9]. During redox cycling, DES is oxidized to DES semiquinone and further oxidized to DES quinone. The quinone is reduced back to hydroquinone (DES) by cytochrome P450 reductase. DES reactive metabolites, quinone and semiquinone covalently attack macromolecules. If covalent modifications in bases of DNA are not repaired, then they may produce mutagenic lesions.

DNA polymerases are not perfect. DNA polymerases insert the wrong base approximately once in every 10^6 bp replicated [10–13]. Additionally, spontaneous oxidative damage to bases of DNA occurs over time [10]. Indigenous mutation rates are estimated at 1 to 10^{12} bp replicated [12]. Since DNA repair is time dependent [10,12], it is reasonable to assume that a reduction in cell cycle time should alter the DNA repair capacity of cells. Recently, we reported that DES stimulates the growth of Leydig cells and reduces cell cycle doubling time [14]. Whether DES directly or through a reduction of cell cycle period attenuates DNA repair capacity of Leydig cells is not clear.

In this study, we used a host cell reactivation assay to investigate the role of cycle time and/or effects of DES on DNA repair capacity [15–17]. This assay uses plasmid DNA with a reporter gene under the control of a specific promoter. When plasmid DNA is transfected into host cells, the reporter gene (e.g. CAT) is expressed. To test the DNA repair capacity of the host cells, the plasmid DNA is first damaged so that it cannot express a functional reporter gene product, which is then transfected into host cells. This method measures the DNA repair capacity as a total process that includes DNA repair activity along with the cell's ability to express the restored gene into a functional protein product. The novel findings emerged from this study are that a reduction in cell cycle time correlated with the lowering of DNA repair; and stilbene estrogen exposure to Leydig cells inhibited DNA repair capacity.

2. Materials and methods

2.1. Materials

TM3-Leydig cells (CRL-1714) were purchased from ATCC, Rockville, MD. Chemicals used were mixed xylenes (Aldrich, Saint Louis, MO); scintillation fluid, [^{3}H] chloramphenicol (DuPont-NEN); PBS buffer (Life Technologies, Grand Island, NY); Tris–HCL, pH 8.0, 2M Ca/Cl_2, and HBS 2× buffer (Promega). The DNA plasmid pCAT® Control plasmid was purchased from Promega. pCAT® plasmid was isolated from *Escherichia coli*. INVαF^- by alkaline lysis method [18]. The culture medium consisting of 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium was purchased from Life Technologies. Diethylstilbestrol (DES) was purchased from Sigma, Saint Louis, MO.

2.2. Cell preparation

Prior to treatment, the cells were cultured using DMEM/F12 medium supplemented with 5% horse serum and 2.5% fetal bovine serum [14]. The growth medium was changed once every 2–3 days. Cultures were subcultured by trypsinization at a ratio of 1:200 when cultures reached 75–80% confluence.

2.3. Host cell reactivation assay

We used the host cell reactivation assay as previously described by Wei et al. (1993) with some modifications [15]. This assay utilizes a plasmid with a reporter gene, which is then damaged by either chemical or physical agents so the reporter gene will not be expressed unless it is repaired. The damaged plasmid is then transfected into host cells and reporter gene expression is monitored over time. We have used Promega's pCAT® Control plasmid. The pCAT® Control Vector contains SV40 promoter and enhancer sequences, resulting in strong expression of CAT [19]. The plasmids were exposed to 350, 700 and 2000 J/m^2 UV-radiation. The exposure of these doses of UV-radiation, shown to induce the formation of thymine dimers, inactivates the expression of CAT gene. These plasmids were designated as UV-damaged plasmids (UVPs). The non-radiated plasmids were considered as undamaged plasmids.

2.4. Plasmid transfection into Leydig cells

3 h before transfection of the plasmids, the growth medium was replaced. The transfection solution

(10 μg DNA, 37 μl CaCl, 263 μl water, and 300 μl Tris buffer per sample) was prepared and incubated at room temperature for 30 min prior to transfection. A total volume of 0.6 ml per sample of transfection solution was added dropwise while swirling the culture flask. Optimal transfection time was determined by monitoring CAT activity every 4 h. Upon completion of the transfection, Leydig cells were washed three times with PBS, and then serum-free medium containing the test compound was added.

Cells transfected with UV-damaged plasmids, -undamaged plasmids (UDP), or no plasmids (sham treatment) were grown in serum, serum-free, or DES plus serum-free medium. Cells were grown for 72 h.

2.5. Cell extract preparation

Cells were lysed with a lysis buffer (1 ml per flask). The cells were incubated for 20 min at room temperature. Cells were dislodged with a cell scraper, and then the extract and cellular debris were collected in Eppendorf tubes. The samples then underwent one freeze–thaw cycle with a minimum of 10 min at 65°C. The tubes were then centrifuged at 10,000 rpm for 3 min. The supernatant was then removed and stored at −70°C until assayed for CAT activity.

2.6. CAT assay

Cell extracts were removed from storage at −70°C and were thawed in a water bath at 65°C for 10 min. The reaction mixture consisting of 70 μl cell extract, 10 μl ^{3}H-chloramphenicol (0.25 μCi), 5 μl *n*-butyl Coenzyme A, and 40 μl distilled water was incubated for 3 h. Termination of the assay was accomplished by briefly spinning the reaction tubes, followed by the addition of 300 μl mixed xylenes. The tubes were then vortexed for 30 s. The organic and aqueous phases were separated by centrifugation at 10,000 rpm for 3 min. Of the aqueous phase, 175 μl was removed, placed into a fresh Eppendorf tube, and a 100 μl Tris buffer 0.25 M, pH 8.0 was added. The tubes were vortexed for 30 s and centrifuged at 10,000 rpm for 3 min. Then, an aliquot of 100 μl of the aqueous phase was transferred into a scintillation vial and 5 ml of scintillation cocktail was added. After vortexing, the samples were counted using a Beckman scintillation counter.

2.7. DNA repair capacity

In order to determine the percent DNA repair capacity, the functional CAT gene activity for the various treatment conditions was converted to a ratio. This was performed by dividing the CAT expression value for the UVP minus the non-specific expression value (NS, obtained from the sham treated flasks) by the expression value for the UDP minus the NS, and multiplied by 100. This formula is shown below:

$$\text{DNA repair capacity (\%)} \cong \text{CAT expression ratio} = \frac{\text{UVP} - \text{NS}}{\text{UDP} - \text{NS}} \times 100$$

The ratio of CAT expression is proportional to the total DNA repair that occurred over time under the specific given condition. This method measures the DNA repair capacity as a total process in which not only the active DNA repair activity is measured, but also the cell's ability to express the restored gene into a functional protein product. The significance of DNA repair activity was analyzed using the Student's *t*-test. The α was set at 0.05 for the Student's *t*-test.

3. Results

Before carrying out DNA repair experiments, validation of transfection was determined by measuring the expression of pCAT® plasmid in Leydig cells grown with serum-free medium over time. The time-dependent increase in the expression of CAT gene after transfection of pCAT® into Leydig cells is shown in Fig. 1. The expression of the CAT gene occurred in a log-phasic fashion in TM3 Leydig cells. Our transfection standardization findings are in agreement with the manufacturer's published sensitivity and limits of detection [19]. To test whether the CAT gene contained within Promega's pCAT® plasmid was sensitive to damage by UV-radiation, we conducted a series of experiments using various doses of UV-radiation (0, 350, 700, or 2000 J/m^2). UV exposure produced a dose-dependent decrease in the expression of CAT gene (Fig. 2).

The metabolic and proliferative activities differ between cells cultured with serum-supplemented medium

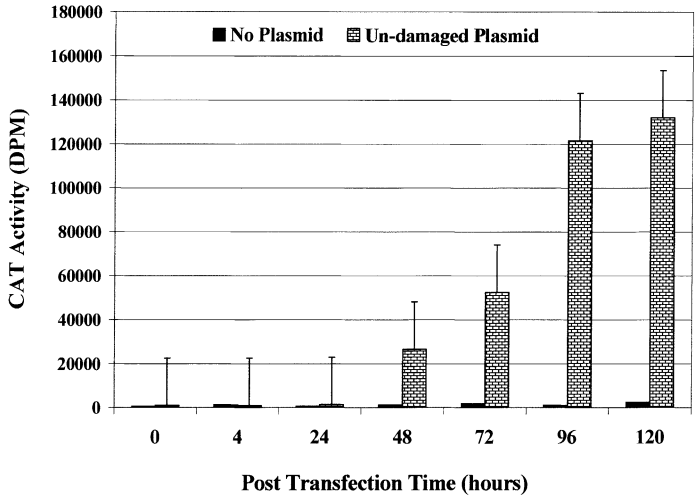

Fig. 1. The time-dependent increase in the expression of CAT gene activity in Leydig cells under serum-free conditions. TM3 Leydig cells were cultured under non hormone-supplemented serum-free conditions [14] and were transfected with a plasmid containing the CAT gene under the control of the SV40 promoter using the manufactures protocol [19]. The CAT activity was measured using Promega's CAT assay kit [19]. Each value is the mean of three independent experiments. The bar represents standard error.

and those cultured with serum-free medium. We compared the DNA repair activity in cells grown in serum-containing and serum-free media. A comparison of CAT expression between serum-free cells and serum grown cells revealed an increase in CAT activity in UV-damaged (45%) and undamaged (79%) plasmid transfected cells grown with serum (Fig. 3). The CAT activity ratio between undamaged and UV-damaged plasmid transfected cells indicates the total DNA repair capacity. The serum grown cells that have a shorter cell cycle time (≅16 h) showed a 40% lower DNA repair capacity when compared to

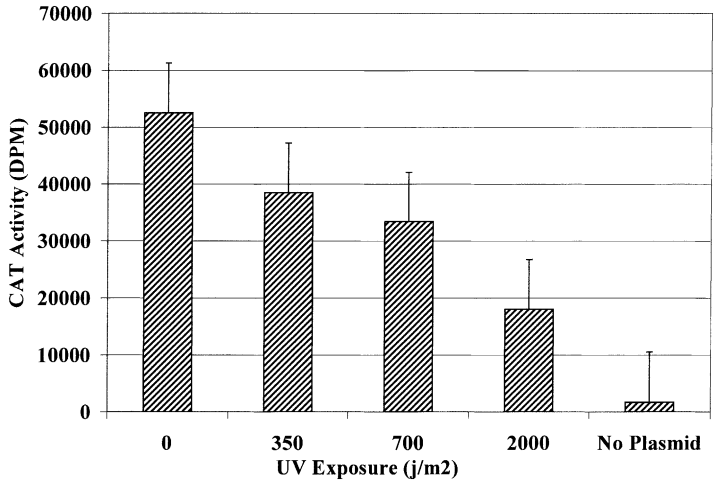

Fig. 2. The effect of different doses of UV-radiation on the expression of the CAT gene activity. TM3 Leydig cells were cultured under non hormone-supplemented serum-free conditions [14]. TM3 Leydig cells were transfected with plasmids exposed to 0, 350, 700, or 2000 J/m^2 UV-radiation. The CAT activity was measured using Promega's CAT assay kit 72 h after transfection [19]. Each value is the mean of three independent experiments. The bar represents standard error.

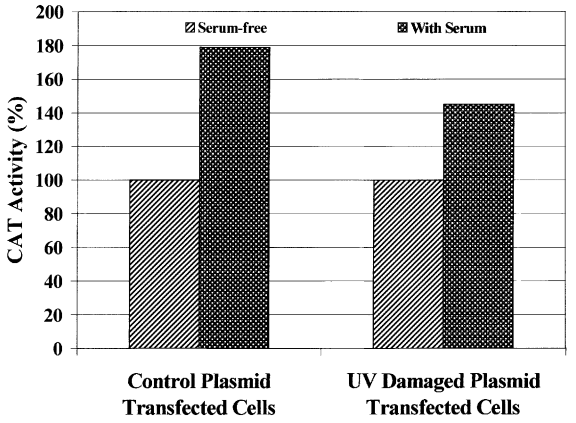

Fig. 3. The effect of serum-supplemented medium on the expression of CAT activity in UV-damaged and undamaged plasmid transfected Leydig cells. TM3 Leydig cells were transfected with plasmids exposed to 0, or 700 J/m^2 UV-radiation. TM3 Leydig cells were cultured with and without serum for 72 h [14]. The CAT activity was measured using Promega's CAT assay kit 72 h after transfection [19]. Each value is the mean of four to six independent experiments.

serum-free cells with a longer cell cycle time (≅25 h) (Fig. 4).

The effect of DES exposure on CAT expression in both UV-damaged and undamaged pCAT® plasmid transfected cells is shown in Fig. 5. The reduction

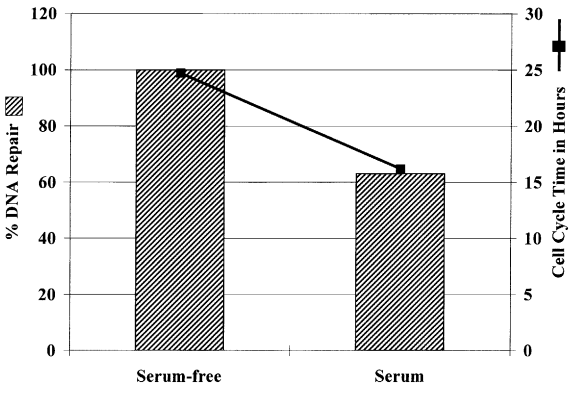

Fig. 4. The effects of serum and serum-free medium on the DNA repair capacity of Leydig cells. TM3 Leydig cells were transfected with plasmids exposed to 0, or 700 J/m^2 UV-radiation. TM3 Leydig cells were cultured with and without serum for 72 h [14]. The DNA repair capacity was calculated as described in the material and method section. Each value is the mean of four to six independent experiments.

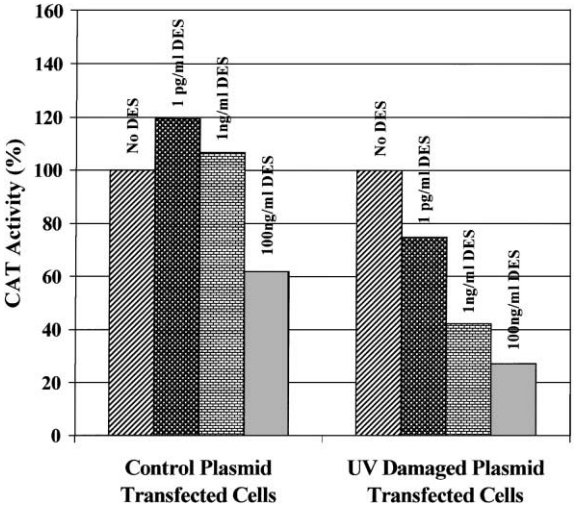

Fig. 5. The effect of DES on the expression of CAT activity in UV-damaged and undamaged plasmid transfected Leydig cells. TM3 Leydig cells were transfected with plasmids exposed to 0, or 700 J/m^2 UV-radiation. TM3 Leydig cells were cultured without serum for 72 h [14]. The CAT activity was measured using Promega's CAT assay kit 72 h after transfection [19]. Each value is the mean of four to six independent experiments.

of CAT activity in UV-damaged plasmid transfected cells by DES occurred in a dose dependent manner. A maximum inhibitory effect was observed with 100 ng/ml (72.87%) followed by 1 ng/ml (57.66%) and 1 pg/ml (25.28%) of DES, respectively. The levels of the CAT activity in undamaged plasmid transfected cells in the presence of DES revealed that 1 pg/ml and 1 ng/ml DES treatment did not alter CAT activity significantly. However, we did observe inhibition in CAT activity by 100 ng/ml DES. A significant decrease in the DNA repair capacity of the Leydig cells exposed to DES was observed when compared to the activity of untreated cells grown in serum-free medium ($P < 0.05$). The DES effect on DNA repair of Leydig cells was dose dependent (Fig. 6). Recently, we reported that DES stimulates the growth of Leydig cells and lowers cell cycle doubling time [14]. When the DNA repair capacity was compared to that of cell cycle doubling time or S-phase cell entry in both untreated and DES treated cells, the observed decreases in DNA repair by DES did not parallel the effects observed on cell cycle kinetics (Fig. 7).

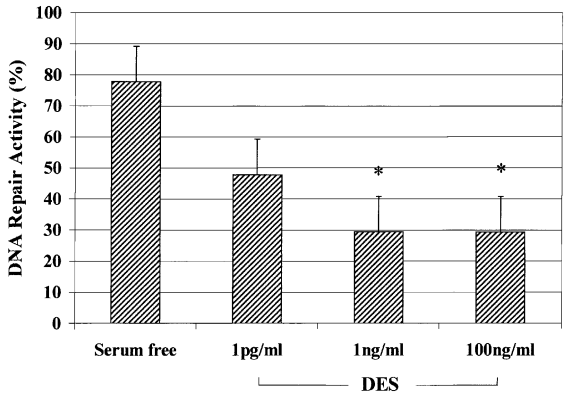

Fig. 6. The effects of DES on the DNA repair capacity of Leydig cells. TM3 Leydig cells were transfected with plasmids exposed to 0, or 700 J/m^2 UV-radiation. TM3 Leydig cells were cultured without serum for 72 h [14]. The DNA repair capacity was calculated as described in Section 2. Each value is the mean of four to six independent experiments. The bar represents standard error. $^*P < 0.05$ indicates significant difference from controls.

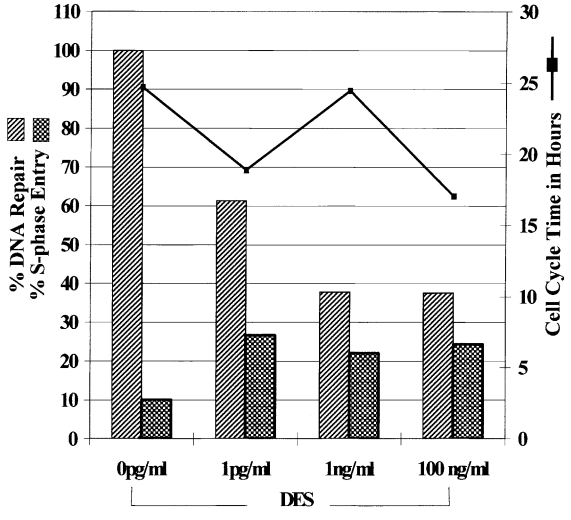

Fig. 7. The correlation of DES induced reduction of DNA repair capacity Leydig cells with changes in cell cycle time and S-phase cell entry. Cell cycle time and S-phase cell entry detection using flow cytometry were determined as described previously [14]. TM3 Leydig cells were transfected with plasmids exposed to 0, or 700 J/m^2 UV-radiation. TM3 Leydig cells were cultured without serum for 72 h [14]. The DNA repair capacity was calculated as described in Section 2. Each value is the mean of four to six independent experiments.

4. Discussion

The major findings of this study are that exposure of Leydig cells to stilbene estrogen inhibited DNA repair capacity, and a reduction in cell cycle time correlated with the lowering of DNA repair capacity in the absence of DES. The data presented here are novel and intriguing as they may provide a molecular mechanism of stilbene estrogen-induced genomic instability in Leydig cells through inhibition of DNA repair capacity. This may also provide a mechanism of incorporation for genetic alterations in Leydig cells by estrogens.

The mechanism of inhibition of DNA repair capacity by stilbene estrogen in Leydig cells is not clear. DES may inhibit the DNA repair system directly through DNA repair gene regulation. For example, DES exposure to Syrian hamster inhibits the mRNA expression level of DNA polymerase β [1]. Since DNA polymerase β is known to play an important role in the DNA repair system, any reduction in the formation of this protein would result in a reduction of DNA repair capacity. In addition, DES can redox cycle and produce reactive metabolites. These DES reactive metabolites may directly inhibit and/or damage the reporter gene (CAT) which, in turn, may inactivate expression of the gene product. We can not rule out that this is a likely mechanism because inhibition of DNA repair capacity by DES was dose dependent. DES mediated reduction in cell cycle time or stimulation of cell proliferation and S-phase cell entry did not correlate with dose dependent inhibition of DNA repair capacity.

Indigenous mutation rates are estimated at 1 to 10^{12} bp replicated [12]. As observed in this study, the addition of DES resulted in a decrease of 44% in DNA repair capacity. If a 44% reduction of DNA repair capacity was assumed, then the average number of somatic cell mutations would be augmented from an average of 1–1.77 per cell cycle. Based on the Hayflick limit, somatic cells can typically divide an average of 50 times in their life span [20]. If an exposure from cell differentiation to cell senescence were assumed, then the total number of mutations associated with replication errors in the normal life span of a cell would increase from approximately 50 to 88. While this is a moderate increase in mutations, it is more significant when it occurs in combination with

ability of DES to induce cell proliferation. This may account for the generation of an adequate amount of alterations in the genome of target organs required for the development of stilbene estrogen tumorigenesis.

5. Summary

The stilbene estrogen DES inhibits Leydig cell DNA repair capacity. The ability of DES to decrease DNA repair may not be solely due to their mitogenic activity. Findings of this study may help in the understanding of the molecular mechanism of genetic instability in Leydig cells induced by natural and environmental estrogens.

Acknowledgements

This research is supported in part by CA52584 (DR). The fellowship support to J.W. DuMond, Ph.D. through the Cancer Treatment and Control Training Program (CA 5R25CA47888-13) is deeply appreciated.

References

[1] D. Roy, M. Palangat, C.W. Chen, R.D. Thomas, J. Colerangle, A. Atkinson, Z.J. Yan, Biochemical and molecular changes at the cellular level in response to exposure to environmental estrogen-like chemicals, J. Toxicol. Environ. Health 50 (1997) 1–29.

[2] R. Bacon, H. Kirkman, The response of the testis of the hamster to chronic treatment with different estrogens, Endocrinol. 57 (1995) 255–271.

[3] W.C. Beckman, R.R. Newbold, C.T. Teng, J.A. McLachlan, Molecular feminization of mouse seminal vesicle by perinatal exposure to DES: altered expression of messenger RNA, J. Urol. 151 (1994) 1370–1378.

[4] T. Colborn, F.S. vom Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ. Health Perspect. 101 (1993) 378–384.

[5] J. Boyd, H. Takalashi, S.F. Waggoner, L.A. Jones, R.A. Hajek, J.T. Wharton, F. Liu, T. Fujimo, J.C. Barret, J.A. MacLachlan, Molecular and genetic analysis of clear cell adenocarcinoma of the vagina and cervix associated and non-associated with DES exposure in utero, Cancer 77 (1996) 507–513.

[6] K.A. Fowler, K. Gill, N. Kirma, D.L. Dillehay, R.R. Tekmal, Over-expression of aromatase leads to development of testicular Leydig cell tumors: an in vivo model for hormone-mediated testicular cancer, Am. J. Pathol. 156 (2000) 347–353.

[7] D.P. Cahill, K.W. Kinzler, B. Vogelstein, C. Lengauer, Genetic instability and Darwinian selection in tumors, Trends Cell Biol. 9 (1999) 57–59.

[8] F. McCormick, Signaling networks that cause cancer, Trends Cell Biol. 9 (1999) 53–56.

[9] D. Roy, J.G. Liehr, Estrogens, DNA damages and mutations, Mutat. Res. 424 (1999) 107–115.

[10] D.I. Feig, L.A. Loeb, Endogenous mutagens, in: J.M.H. Vos (Ed.), DNA repair mechanism: impact on human diseases and cancer, R.G. Landes Company, Austin, TX, 1995, pp. 175–185.

[11] L. A Loeb, T. A Kunkel, Fidelity of DNA synthesis, Ann. Rev. Biochem. 52 (1981) 429–457.

[12] E.C. Friedberg, G.C. Walker, W. Siede, DNA damage, in DNA repair and mutagenesis, Am. Soc. Microbiol., Washington, DC, 1995, pp. 1–58.

[13] L.A. Loeb, L.A. Weymouth, T.A. Kunkel, K.P. Gopinathan, R.A. Beckman, D.K. Dube, On the fidelity of DNA replication, Quant. Biol. 43 (1978) 921–927.

[14] J.W. DuMond, D. Roy, The biphasic stimulation of proliferation of Leydig cells by estrogen exposure, Int. J. Oncol. 18 (2001) 623–628.

[15] Q. Wei, G.M. Matanoski, E.R. Farmer, M.A. Hedayati, L. Grossman, DNA repair and aging in basal cell carcinoma: a molecular epidemiology study, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 1614–1618.

[16] L. M Hallberg, W.E. Bechtold, J. Grady, M.S. Legator, W.W. Au, Abnormal DNA repair activities in lymphocytes of workers exposed to 1,3-butadiene, Mutat. Res. 3838 (1997) 213–221.

[17] M. D'Errico, A. Calcagnile, I. Iavarone, F. Sera, G. Baliva, L.M. Chinni, R. Corona, P. Pasquini, E. Dogliotti, Factors that influence the DNA repair capacity of normal and skin cancer-affected individuals, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 553–559.

[18] J. Sambrook, E.F. Frisch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

[19] Promega Protocols and Applications Guide, 2nd Edition, Promega Corporation, Madison, WI, 1991.

[20] L. Hayflick, The limited in vitro lifetime of human diploid cell strains, Exp. Cell Res. 37 (1965) 614–636.