REACTIVE OXYGEN SPECIES-INDUCED TOXICITY AND MUTANTS IN CHINESE HAMSTER OVARY CELLS

INDUCCIÓN DE TOXICIDAD Y MUTANTES POR ESPECIES REACTIVAS DE OXÍGENO EN CÉLULAS DE OVARIO DE HÁMSTER CHINO

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Abstract

The chinese hamster ovary (CHO) cell system is widely used system to study spontaneous and induced-toxicity and mutagenicity in mammalian cells. By comparing toxicity and mutation spectrum of identically exposed repair proficient and repair-deficient cell lines, the possible roles of DNA repair in toxicity may be defined. In this study we propose that parental CHO-K1-BH4 is more resistant to oxidative damage caused by hydrogen peroxide (H_2O_2), added directly to the medium or generated steadily by glucose oxidase (GO), paraquat and xanthine oxidase (XO) than its derivatives AS52 and XRS-5. We hypothesize that the various CHO cell lines differ in sensitivity to oxidative damage and that the observed toxicity may be due to accumulation of DNA breaks and impairment of the mitochondrial functions. High toxicity was observed (low clonogenic survival) for all tested compounds in all cell lines. Line XRS-5 seemed to be more sensitive to reactive oxygen species (ROS)-induced toxicity. CHO-K1-BH4 parental cell line appeared to be more resistant to the compounds studied when compared to the radiosensitive XRS-5 cell line. Glucose oxidase (4 mU/ml) resulted equitoxic to parental and derivatives cell lines. 500 mM of H_2O_2 increased ~4 fold the mutant frequency above the background in AS52. Xanthine oxidase (100 mU/ml) increased ~4 fold the mutagenicity frequency in XRS-5. None of the other tested compounds induced 6-TG resistance as mutagenic response. This difference may be due to different ability to handle oxidative damage associated with genetic composition and repair enzymatic responses.

Key words: CHO cells, oxidative damage, mt toxicity, mtDNA damage.

Resumen

Las células de ovario de hámster chino (CHO) se han usado ampliamente para evaluar toxicidad y mutagenicidad espontánea e inducida. Comparando la toxicidad y el espectro mutacional de líneas celulares eficientes y deficientes en reparación, se podría definir la relación entre toxicidad y reparación del DNA. En este estudio se propone que la línea parental CHO-K1-BH4 es más resistente que sus líneas derivadas AS52 y XRS-5 al daño oxidativo causado por peróxido de hidrógeno (H_2O_2), agregado directamente al medio o generado constantemente por glucosa oxidasa (GO), paraquat o xantina oxidasa (XO). Nuestra hipótesis es que las líneas celulares CHO difieren en sensibilidad al daño oxidativo y que la toxicidad observada puede ser debida a la acumulación de roturas en el DNA y al deterioro de las funciones mitocondriales. Todos los compuestos estudiados produjeron alta toxicidad (baja sobrevivencia clonogénica) en todas la líneas celulares evaluadas. La línea XRS-5 parece ser más sensible a la toxicidad inducida por especies reactivas de oxígeno (ROS). La línea celular parental, CHO-K1-BH4, es más resistente a los compuestos evaluados si se compara con su derivada radiosensitiva, XRS-5. La glucosa oxidasa (5 mU/ml) fue equitóxica tanto a la línea celular parental como a sus derivadas. El Peróxido de hidrógeno (500 mM) aumentó aproximadamente cuatro veces la frecuencia de mutantes en la línea celular AS52. La xantina oxidasa (100 mU/ml) aumentó cerca de cuatro veces la frecuencia mutagénica en XRS-5. Ninguno de los otros compuestos probados indujo resistencia a la 6-TG como una respuesta mutagénica. Esta diferencia puede ser debida a la capacidad de manipulación del daño oxidativo de cada línea celular, asociada a su composición genética y su respuesta enzimática reparativa.

Palabras claves: células CHO, daño oxidativo, mt toxicidad, mtDNA daño.

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INTRODUCTION

Since the development of the CHO/HGPRT protocol (O'Neill *et al.*, 1977), CHO cell lines have been widely used to study toxicity and mutagenicity in a number of physical and chemicals agents (An and Hsie, 1992; An and Hsie, 1993; Aruoma *et al.*, 1989a; Aruoma, *et al.*, 1989b; Baggetto, 1993; Blunt *et al.*, 1995; Chance *et al.*, 1979; Chandrasekhar and Van Houten, 1994). The system is based on mutations induced at the HGPRT locus in CHO cells by using resistance to 6-TG as a marker to recognize HGPRT- mutants, allowing the evaluation of damage on a gene-specific basis (Aaron and Stankowski, 1989). Thioguanine (TG) is lethal to normal mammalian cells and resistance to this purine analogue results from an alteration or loss of the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRTase). The enzyme-competent wild type cells do not form colonies because they are able to convert TG to nucleotide thioguanine monophosphate and are killed as a result of accumulation of toxic TG metabolites (Siminovitch, 1976). The introduction of mutant CHO-K1 derivatives such as AS52 (Stankowski and Hsie, 1986; Mulligan and Berg, 1980) (containing an *E.coli gpt* gene) and XRS-5 (Jeggo and Kemp, 1983; Kemp *et al.*, 1984) (radiation sensitivity due to strand break repair deficiency) to the CHO system has made the protocol more sensitive and useful to study reactive oxygen species (ROS)-induced mutagenicity and toxicity (Hsie *et al.*, 1986; Oller and Thilly, 1992; Ziegler-Skylakakis and Andrae, 1987; An and Hsie, 1992; Yu *et al.*, 1994; Zdzienicka, 1995). The use of wild type and derivative mutants permits comparison of various cell lines to study gene-specific sensitivity on a given agent. The selection of mutants, combined with recently developed molecular biology protocols, allows for screening of deletions and specific mutations at the *hprt*, *gpt* and *xrcc5* genes (Hsie *et al.*, 1993; An and Hsie, 1993), greatly improving the CHO system. Reactive oxygen species are a product of normal cellular metabolism (Chance *et al.*, 1979).

Hydroxyl groups are mainly responsible for the observed damage and are generated in the presence of hydrogen peroxide and iron clusters, through the Fenton reaction (Luo *et al.*, 1994; Henle and Linn, 1997). The hydroxyl groups could then react with critical cellular molecules such as DNA causing strand breaks (Spragg, 1991), abasic sites, and base modification (Halliwell and Aruoma, 1991; Meneghini,1988).

Besides 8-hydroxydeoxyguanosine, glyoxal is a major product of DNA oxidation in which the Fenton-type reaction is involved through the free radical forming system. The plasmid pMY189 treated with glyoxal and transfected into mammalian cells demonstrated that the majority of glyoxal-induced mutations are single-base substitutions, mainly at G:C base pairs (Kamiya-Murata *et al.*, 1997). Oxidation of proteins is also a consequence of oxidant exposure (Davies, 1993). Lipids are major constituents of cellular membranes, therefore lipid peroxidation is an important damage occurring at the membrane level and associated with oxidant exposure (Hruszkewycz, 1988). Due to the presence of iron-copper clusters in the mitochondrial proteins, mainly transport electron chain proteins which might mediate the Fenton reaction, is believed that mitochondria are a preferential target of hydrogen peroxide and hydroxyl groups (Aruoma *et al.*, 1989; Ritcher, 1992; Hegler *et al.*, 1993; Yakes and Van Houten, 1997; Salazar and Van Houten, 1997).

Although, chronic exposure to ROS has been associated with several neurodegenerative human diseases, cancer and aging (Koller, 1997; Schapira, 1993; Mizuno *et al.*, 1993; Baggetto, 1993; Sohal *et al.*, 1995; Sohal and Weindruch, 1996) , the mechanisms remain to be elucidated.

Hydrogen peroxide *hprt*-induced and spontaneous mutations studies have been conducted with a TK-6 lymphoblastoid cell line (Oller and Thilly, 1992). Hydrogen peroxide treatment increased the frequency of 6-thioguanine-resistant clones in V79 CHO cells (Ziegler-Skylakakis and Andrae, 1987).

Exogenous ROS-generators include industrial toxicants, cigarette smoke, asbestos, and herbicides such us paraquat, which have been used as models of superoxide generation (Quinlan *et al.*, 1994; Wang *et al.*, 1992; Taylor *et al.*, 1993; Sofuni and Ishidate Jr., 1988).

Glucose in the presence of glucose oxidase has been shown to be a suitable system for steady production of hydrogen peroxide mimicking chronic exposure to a desirable dose of hydrogen peroxide (Spragg, 1991; Salazar and Van Houten, 1997). The xanthine/ xanthine oxidase system also is a widely system used to generate H_2O_2 and superoxide anion (Aruoma *et al.*, 1989a).

In this study we propose that parental CHO-K1-BH4 is more resistant than its derivatives AS52 and XRS-5 to oxidative damage caused by H_2O_2 (added directly to the medium or generated steadily by GO), paraquat and XO.

MATERIALS AND METHODS

Description of cell lines. K1-BH4, AS52 and XRS-5 cell lines were used for toxicity and mutagenicity experiments. CHO-K1-BH4 is proline auxotrophic with a modal chromosome number of 20, which grows in monolayer in Ham's F-12 medium containing 5% FBS. Population doubling has been stated to be 12-14 hours. Cloning efficiency is usually greater than 80% (Hsie *et al.*, 1981). AS52 cells, derived from K1-BH4 with an *hprt* gene deletion, contain a single stable and functional copy of a transfected *E. coli gpt* gene located on an autosome, presumably chromosome 6 (Stankowski and Hsie, 1986). The CHO, XRS-5 mutants are sensitive to ionizing radiation, defective in DNA double-strand break rejoining, and unable to carry out V(D)J recombination effectively. The gene *XRCC5*, defective in these mutants, has been shown to encode Ku80, a component of the Ku protein and DNA-dependent protein kinase (Jeggo and Kemp, 1983; Kemp *et al.*, 1984; Blunt *et al.*, 1995).

Ku protein is an abundant nuclear protein associated with DNA-end binding, one of the steps involved in repairing DNA strand breaks, identified originally as an autoantigen recognized in sera from various autoimmune patients. Ku protein is a heterodimer complex containing two subunits: one with a molecular weight of approximately 70 kD (Ku70) and the other of aproximately 80 kD (Ku80). This protein complex binds specifically to the ends of DNA duplexes. Recently, Ku80 has been identified as the product of gene *XRCC5*. Cells of this complementation group lack DNA-end binding activity (reviewed in Zdzienicka, 1995).

For routine maintenance and passing, all cell lines cells were kept at 37 °C, 5% CO_2 and 100% humidity.

Selection of HPRT$^-$ and GPT$^-$ mutants. To decrease the background of HPRT$^-$ mutants, K1-BH4 and XRS-5 cells were cultured for 48 hours in medium F-12, 5% FBS, supplemented with 100 mM hypoxanthine, 10 mM aminopterin and 10 mM thymidine (HAT medium). To decrease the background of GPT$^-$ mutants, AS52 cells were grown in F-12 medium, 5% FBS, supplemented with 250 mg/ml xanthine, 25 mg/ml adenine, 5 mM thymidine, 3 mM aminopterin and 10-20 mg/ml mycophenolic acid (MPA medium) for 48 hours. After 48 hours recovery in medium F-12, 5% FBS, cells were plated for chemical treatment.

Chemical treatment and doses. Experiments to determine the doses and conditions for toxicity and mutagenicity studies of three cell lines (K1-BH4, AS52 and XRS-5) were carried out. Several doses of H_2O_2, added directly to the medium (0-2 mM) or generated by the glucose / glucose oxidase system (0-50 mU/ml), paraquat (0-2 mM), and xanthine oxidase (0-100 mU/ml) were used.

Aproximately $5x10^5$ cells were plated for chemical treatment. Fresh working solutions of compounds to test were prepared in F-12 serum-free medium immediately before treatment to avoid loss of activity.

The total volume of treatment was 5 ml to assure full coverage of the monolayer's surface. In the control group (without treatment), only 5 ml of F-12

serum-free medium were added. Length of treatment was two hours in all the experiments. Cells were then washed twice with PBS and incubated 24 hours for recovery. After recovery, cells were counted and plated for cloning efficiency and phenotypic expression as described below.

Toxicity / mutagenesis and selection for resistance to 6-TG. All cell culture, mutagenesis and selection experiments for mutants resistant to 6-thioguanine (6-TG^r) were performed as described by O'Neill *et al.* (1977). Briefly, CHO cells were plated at $5x10^5$ cells/100-mm dish in F-12 medium supplemented with 5% fetal bovine serum (FBS). After a 24 hour growth period (cell number ~1.0-1.5 $x10^6$ cells/plate), the chemical agent to be tested was added to the medium and the cultures were incubated for two hours. Cells were washed with PBS once before and twice after treatment. Cells were allow to recover during a 24 hour period after treatment. The number of cells were determined and 200 cells plated in 60-mm dishes in regular medium in triplicate for each culture. These cells were incubated for 7-10 days, the surviving colonies counted, and the relative cell survival (cloning efficiency) was determined and expressed as relative percentage of the untreated control dishes, which usually yielded an efficiency of 80% or better.

Another set of treated cells (10^6, or all remaining if $< 10^6$) were also plated in 100-mm dishes in regular medium for phenotypic expression. For determination of mutant induction (expression of the TG^r phenotype), the cells plated in 100-mm dishes, were subcultured every 48-72 hours in regular medium. Depending on the extent of cell survival after chemical treatment, one or two subcultures were performed during this period.

After 7 or 8 days of culture, cells were plated for selection in the presence of 6-TG. The number of cells was determined. For cloning efficiency measurements, 200 cells were plated into 60-mm dishes, triplicate, in hypoxanthine (HX)-free F-12 medium containing 5% dialyzed fetal serum. For measurements of TG resistance, $2x10^5$ cells were plated into 100-mm dishes (5 plates = $1x10^6$ cells total plated) in F-12 HX$^-$ medium, containing TG at final concentration of 10 mM.

After 7-10 days of incubation, grown colonies were scored and the mutant frequency (MF) was calculated by dividing the total number of mutant colonies grown in the TG medium (TG^r mutants) by the total number of cells plated ($1x10^6$ clonable cells), corrected by the cloning efficiency.

Colonies were then isolated by the ring method. The isolated colonies were expanded for further DNA isolation and molecular analyzes. These independently derived mutants, 6-TG^r colonies, were isolated by trypsinization in a stainless steel ring sealed to the bottom of the dish with autoclaved silicone lubricant. The isolated mutant colonies were then grown to approximately $2x10^6$ cells in medium F-12, 5% FBS. Finally, cells were frozen until DNA isolation is performed for further molecular mutagenesis screening.

RESULTS

The results obtained indicate that CHO is a good system to evaluate ROS-induced toxicity and mutagenicity in a specific gene at the nuclear level. High toxicity was observed (low number of colony survival) for all tested compounds in all cell lines (fig. 1, panels A, B, C, D) in a dose-dependent manner. Toxicity data expressed as LD_{50} (the lethal dose needed to kill 50% of the exposed cells) appears in table 1. The XRS-5 cell line is the most sensitive to ROS-induced toxicity if compared to the other two cell lines. The CHO parental cell line is more resistant to H_2O_2 and paraquat when compared to radiosensitive XRS-5 and AS52 cell lines. Glucose oxidase (4 mU/ml) is equitoxic to parental and derivative cell lines (fig. 1, panel C).

Relatively low doses of the compounds (400 µM H_2O_2, 2 mU/ml GO and 200 µM paraquat) are not toxic to AS52. X/XO was tested only with the XRS-5 cell line, showing high toxicity to doses as low as 10 mU/ml (fig. 1, panel D).

gpt gene is more sensitive to ROS-induced mutagenesis. Although there is a high mutant frequency background in AS52 cells, 500 µM of H_2O_2 increased ~4 fold the mutant frequency (fig. 2, panel A). None of the other tested compounds induced 6-TG resistance as a mutagenic response. There was no increased mutation frequency for the parental K1-BH4 or the radiosensitive XRS-5 cell lines.

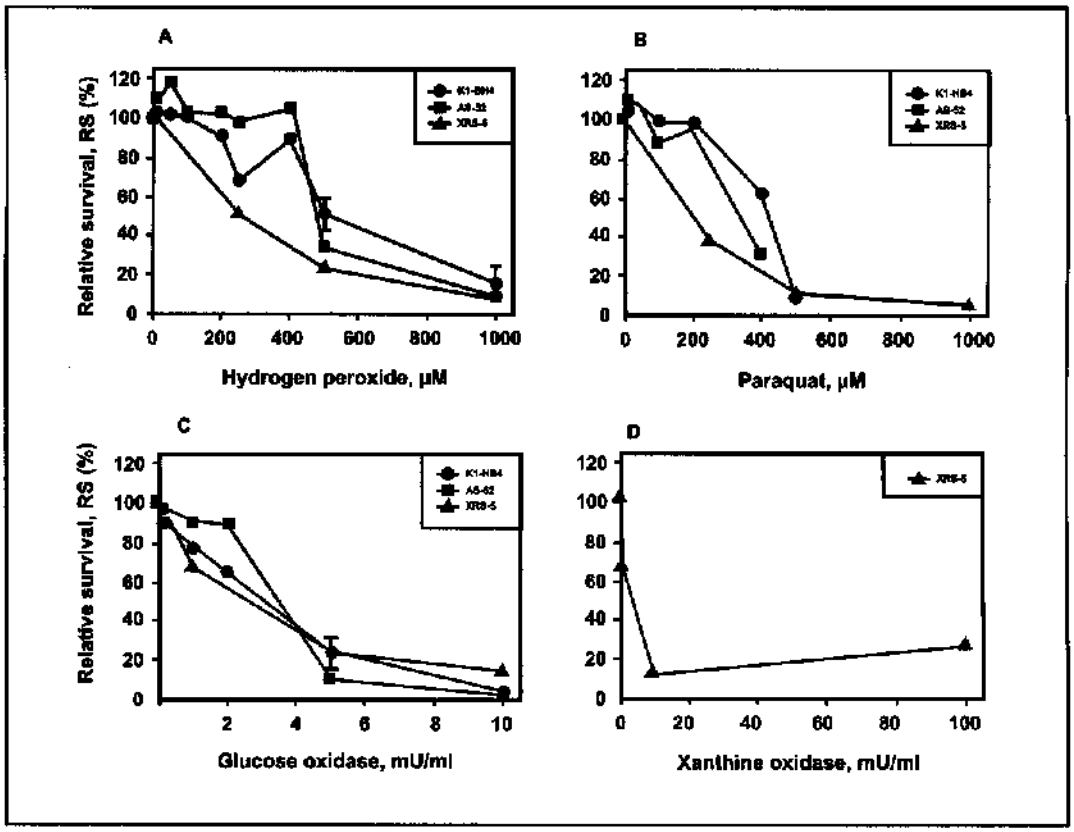

Figure 1. Cytotoxicity response of CHO cell lines exposed to ROS-generators. The cytotoxicity is measured by the cloning efficiency (CE) and expressed as relative survival (RS) of the untreated control (100 %). Panels A, B, C and D show each compound and concentrations used to generate toxicity. Each point represent at least one experiment from triplicate dishes.

Table 1. LD_{50} of four ROS generators in three CHO cell lines. Cytotoxicity data expressed as LD_{50} for four ROS generators in several CHO cell lines.

ROS generator	K1-BH4	AS52	XRS-5
H_2O_2 (µM)	500	480	260
Paraquat (µM)	440	340	200
GO (mU/ml)	3	3.5	2.5
XO (mU/ml)			4

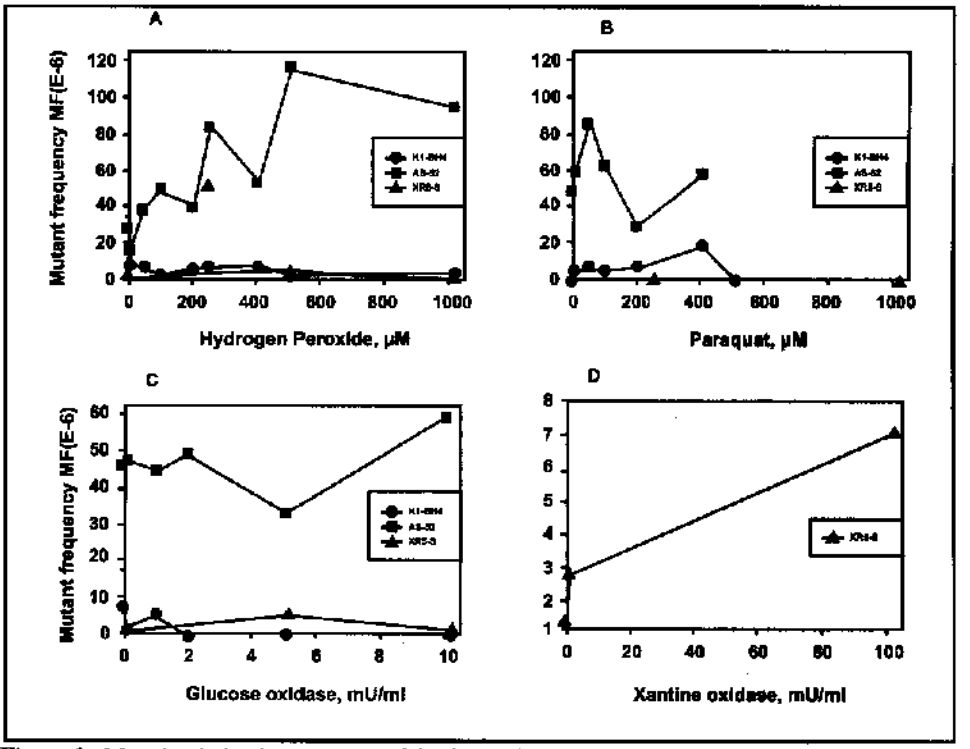

Figure 2. Mutation induction response of CHO cell lines exposed to ROS-generators. The mutant frequency is expressed as MF (TG^r mutants/10^6 clonable cells) corrected by cloning efficiency (CE). Panels A, B, C, and D show each compound and concentrations used to induce mutants. Each point represent at least one experiment from triplicate dishes.

DISCUSSION

The purpose of this study was to use the CHO system to evaluate ROS-induced toxicity and mutagenicity. All cell lines showed a decreased ability to form colonies after exposure to various doses of oxidative compounds in a dose dependent manner.

Cytotoxicity data expressed as LD_{50} indicated that there was a higher toxicity in XRS-5 cells exposed to H_2O_2, paraquat and GO, as compared to repair efficient K1-BH4 and AS52 cells. These findings suggest that the accumulation of unrepaired ROS-induced DNA breaks may be implicated in lethality. Hydrogen peroxide added directly to the medium increased the mutation frequency ~4 fold above the background level in AS52, and XO increased two fold the mutation frequency in XRS-5 cells.

Glucose oxidase is equitoxic to parental and derived cell lines when treated with 4 mU/ml. Hsie *et al.* (1993) found that X-irradiation is equally toxic to both AS52 cells and the parental K1-BH4 cells. In spite of the observed equitoxicity, the mutant frequency in the *gpt* locus was several fold higher when compared to the *hprt* locus in parental K1-BH4 cells. The observation that H_2O_2 increased the mutagenic background in the *gpt* locus in AS52 cells has been previously shown by others (Hrelia and Hsie, 1989).

CHO-K1-BH4 and AS52 cells exhibited similar cytotoxicity response to UV light and X-rays. Conversely, significant differences were observed when the mutagenicity was evaluated between the *gpt* and *hprt* genes (Stankowski and Hsie, 1986).

The *gpt* locus is more sensitive to adriamyc in-induced mutagenicity. Yu *et al.* (1994) showed that 63% of Adriamycin-induced mutants presented a total deletion of the *gpt* gene.

We did not observe increased mutation frequency in the parental CHO-K1 cells, probably due to an ability

to repair the damage generated by the tested compounds. Future studies will address repair capability of ROS-induced DNA damage in CHO-K1 cells and its derivatives.

There are a number of lines evidence pointing towards the mitochondria as a main target for ROS-induced damage (Luo *et al.*,1994; Henle and Linn, 1997; Ritcher, 1992; Hegler *et al.*, 1993; Yakes and Van Houten, 1997; Salazar and Van Houten, 1997). Mitochondria might be at a disadvantage compared to the nucleus due to lack of histones (which might help to protect nuclear DNA from damage), abundant iron-copper clusters (which favor Fenton reaction and generation of hydroxyl groups) and some repair pathways may not be as efficient in mitochondria as they are in the nucleus. Although CHO cell lines represent a suitable model to study ROS-induced toxicity and mutagenicity, the number of published studies do not satisfy the explanation for the observed toxicity.

In conclusion, our results indicate that CHO is a good system to evaluate toxicity and mutagenicity in a gene-specific manner at the nuclear level. In addition, it is important to understand the mechanisms of the observed toxicity. Our goal is to initiate experiments based on XLQPCR protocols (Yakes and Van Houten, 1997; Salazar and Van Houten, 1997; Chandrasekhar and Van Houten, 1994; Yakes *et al.*, 1996) which will allow us to amplify the entire mitochondrial genome for comparison with other genes to test the hypothesis that mitochondria are the main target for ROS in mammalian cells.

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