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Protection against Radiation-Induced Mutations at the *hprt* Locus by Spermine and *N,N''*-(Dithiodi-2,1-ethanediyl)bis-1,3-Propanediamine (WR-33278)[1]

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ABSTRACT

The polyamine spermine and the disulfide *N,N''*-(dithiodi-2,1-ethanediyl)bis-1,3-propanediamine (WR-33278) are structurally similar agents capable of binding to DNA. WR-33278 is the disulfide moiety of the clinically studied radioprotective agent S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721). Because of their reported structural and functional similarities, it was of interest to characterize and compare their radioprotective properties using the endpoints of cell survival and mutation induction at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus in Chinese hamster AA8 cells. In order to facilitate both the uptake of WR-33278 into cells and the direct comparison between the protective properties of WR-33278 and spermine, these agents (at concentrations of 0.01 mM and 0.001 mM) were electroporated into cells. Electroporation, 300 V and 125 μFD, was performed either 30 min prior to or 3 h following exposure of cells to 750 cGy of ionizing radiation. Electroporation alone reduced cell survival to 75% but had no effect on *hprt* mutation frequency. The electroporation of either spermine or WR-33278 at concentrations greater than 0.01 mM was extremely toxic and, therefore, precluded the study of higher concentrations of these agents. The exposure of cells to both electroporation and irradiation gave rise to enhanced cell killing and mutation induction, with the sequence of irradiation followed 3 h later by electroporation being the more toxic protocol. Enhanced cell survival was observed following electroporation of 0.01 mM of spermine and WR-33278 30 min prior to irradiation; protection factors (PF) of 1.3 and 1.8, respectively. Neither agent was protective at a concentration of 0.001 mM. Protection against radiation-induced *hprt* mutations

was observed for both spermine and WR-33278 under all experimental conditions tested. Spermine at concentrations of 0.01 mM and 0.001 mM administered 30 min before or 3 h after irradiation produced PFs of 2.2, 1.2, 1.9 and 2.2, respectively. WR-33278 at concentrations of 0.01 mM and 0.001 mM administered 30 min before or 3 h after irradiation produced PFs of 1.8, 1.3, 1.4 and 2.0, respectively. The close agreement in the magnitudes of protection afforded by spermine and WR-33278 against both radiation-induced cell killing and mutagenesis is consistent with their known structural and functional similarities. These data suggest that the properties of radioprotection and chemoprevention exhibited by the phosphorothioate (WR-2721) and associated aminothiol (WR-1065) and disulfide (WR-33278) metabolites may be mediated via endogenous spermine-like polyamine processes. Such a mechanism would have important implications with respect to the design and development of new generation drugs for use in radioprotection and chemoprevention.

INTRODUCTION

The phosphorothioate WR-2721[3] has been investigated in a number of clinical trials as a radiation (1,2) and chemical (3–5) protector. Early studies have suggested that WR-2721 is highly effective in protecting normal cells from the lethal effects of radiation and chemotherapy but is relatively ineffective in protecting tumor cells (6,7). Thus, it has been proposed that WR-2721 would be a useful adjuvant for selected cancer therapies, since it would confer a differential protection to dose-limiting normal tissues.

Interest in this agent has been further stimulated by reports that it possesses anticarcinogenic (8,9) and antimutagenic (10,11) properties. Protection against the induction of mutations at the *hprt* locus in mouse T splenocytes has been reported for WR-2721, even when it was administered to animals up to 3 h following irradiation (11).

WR-2721 is a prodrug which requires dephosphorylation before its protective properties are observable. Following dephosphorylation with alkaline phosphatase, both free thiol (WR-1065) and disulfide (WR-33278) moieties are formed (12), with the thiol form being the most closely correlated with protection against cell lethality (13).

The phosphorothioate WR-2721 and its free thiol WR-1065 and disulfide WR-33278 are structurally similar to polyamines. WR-2721 has been shown to be effective in competitively inhibiting the uptake of putrescine into rat lung tissue (14) as well as being a substrate for polyamine oxidase (15). When compared to polyamines having the same net charge, both the disulfide WR-33278 and free thiol WR-1065 are found to bind to DNA in a similar fashion (16,17). WR-33278 has a binding site of approximately ten nucleotides, while WR-1065 has a binding site of about five nucleotides (16). WR-33278 has also been reported to be capable

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[3]The abbreviations used are: WR-2721, S–2–(3–aminopropylamino)ethylphosphorothioic acid; WR-1065, 2–[(aminopropyl)amino]ethanethiol; WR-33278, *N,N"*-(dithiodi-2,1-ethanediyl)bis-1,3-propanediamine; *hprt*, hypoxanthine-guanine phosphoribosyl transferase; PBSA, phosphate buffered sucrose; PF, protection factor.

of stimulating topoisomerase-I-mediated unwinding of supercoiled DNA (18) in a manner similar to that described for the polyamine spermine (17).

The close structural and functional similarities between these two classes of agents suggests that they may also possess similar activities with regard to their ability to protect against radiation-induced lethality and mutagenesis. To assess this possibility, the polyamine spermine and the disulfide WR-33278, two structurally similar agents, were compared as to their respective abilities to protect mammalian cells from radiation-induced mutagenesis at the *hprt* locus and cell killing.

MATERIALS AND METHODS

Cells and Culture Conditions

Chinese hamster (CHO) AA8 cells were maintained as stock cultures in α–Minimal Essential Medium (Gibco) with 10% fetal calf serum (Biologos) in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. All cell cultures were treated with medium containing hypoxanthine (5×10^{-5} M), aminopterin (3.2×10^{-6} M), and thymidine (5×10^{-6} M) for 24 h to reduce the background of spontaneous *hprt* mutations.

Drug Treatment

WR-33278 used in these studies was a gift from Dr. C. E. Swenberg, Radiation Biochemistry Department, Armed Forces Radiobiology Research Institute, Bethesda, MD (18). Spermine, anhydrous, was obtained from Sigma Chemical Co., St. Louis, MO (lot 59F07367). Both agents were made up fresh for each experiment in PBSA (7 mM KH_2PO_4; 1 mM $MgCl_2$; and 272 mM sucrose) at pH 7.4 to concentrations of 0.01 mM and 0.001 mM.

Electroporation

Because of the inability of the disulfide WR-33278 to be taken up by cells (12), it was necessary to introduce the drug into cells by the method of electroporation. To facilitate a

comparison between the protecting properties of WR-33278 and spermine, both agents (at concentrations of 0.01 mM or 0.001 mM) were electroporated into cells following a method described in detail elsewhere (19). In each instance, 5×10^6 cells were suspended into 800 μl of ice cold PBSA and added to a 0.4 cm electroporation cuvette. Electroporation was performed using a Bio-Rad Gene Pulser, Richland, CA. After electroporation, samples were held on ice for 30 min and resuspended into 15 ml centrifuge tubes containing 5 ml of medium. The relative efficiency of transfer of material into cells by electroporation was determined using β–galactosidase as a marker. After electroporation, β–galactosidase activity was measured in cells by the method of Winegar and Lutze (20) using histological staining. β–Galactosidase activity was detected in 100% of the electroporated cells measured but was not observed in control cells which were not exposed to electroporation.

Survival and Mutation Studies

Exponentially growing AA8 cells were irradiated in air with ^{60}Co γ rays from a γ-beam 650 irradiator (Atomic Energy of Canada) at a dose rate of 0.5 Gy/min, either 30 min following or 3 h prior to electroporation. Radiation conditions included the exposure of non-electroporated control cells as well as cells electroporated in the presence of 0.01 mM or 0.001 mM spermine or WR-33278. Cell survival was determined by plating appropriate numbers of cells to give between 80 and 200 colonies per dish, 6 dishes per experimental point. Mutation induction at the *hprt* locus was assayed by seeding at least 10^6 surviving cells per experimental point into roller bottles for culture for 9 days to allow for expression (21). To determine mutation frequencies, cells were exposed to 6-thioguanine (Sigma Chemical Co., St. Louis, MO) at a concentration of 5 μg/ml in nucleoside-free α-MEM-10 medium for 7 days and then stained with 0.5% methylene blue. Mutation frequency was expressed as mutants per 10^6 cells following a correction for the cloning efficiency at the time of selection. All experiments were repeated 3 times. Error bars were determined and

represent standard error of the mean. Comparisons of data sets were performed using student's two-tailed *t* test.

RESULTS

Electroporation Effects on CHO AA8 Cells Exposed to WR-33278 and Spermine

Electroporation conditions were developed empirically so as to minimize toxicity while facilitating the cellular uptake of WR-33278 and spermine. Using electroporation conditions of 300 V and 125 μFD, cell survival was reduced to about 74% of untreated controls. Electroporation of either WR-33278 or spermine into cells did not, however, affect their viability (Fig. 1*A*). In contrast to the effect of electroporation on cell survival, no effect was observed by this procedure on either the direct induction of *hprt* mutants or the induction of *hprt* mutants in cells exposed to WR-33278 or spermine (Fig. 1B). Electroporation of WR-33278 or spermine at concentrations greater than 0.01 mM was toxic to cells. All experiments were, therefore, limited to drug concentrations of 0.01 mM and 0.001 mM.

Fig. 1 →

Electroporation Effects on the Radiation Response of CHO AA8 Cells

The procedure of electroporation significantly affected the response of CHO cells to radiation exposure. At a dose of 750 cGy, cell survival was reduced to 2.9% ± 0.3%. When cells were electroporated in PBSA and then irradiated 30 min later, cell survival was reduced to 2.0% ± 0.2%. The most lethal effect was observed when cells were irradiated first and then subjected to electroporation 3 h later (Fig. 2*A*). Under these conditions cell survival was reduced to 1.5% ± 0.1%. Mutation induction in irradiated cells was also significantly enhanced if cells were also subjected to electroporation (Fig. 2*B*). The sequence of electroporation versus irradiation did not, however, significantly affect mutation induction. A dose of 750 cGy gave rise to 75 mutants per 10^6 surviving cells, as compared to 155 and 166 per 10^6 surviving cells for cells electroporated before or following irradiation, respectively.

Fig. 2 →

WR-33278 and Spermine Effects on Radiation Response

When contrasted to the level of response of cells exposed to electroporation and 750 cGy of radiation, cells electroporated with 0.01 mM WR-33278 prior to irradiation exhibited a somewhat enhanced survival level (Fig. 3*A*). WR-33278 was ineffective as a radioprotector when administered at the lower concentration of 0.001 mM or when it was administered following irradiation (Fig. 3*B*). WR-33278, at a concentration of 0.01 mM administered prior to irradiation, increased the surviving fraction from 2 ± 0.2% to 3.7 ± 0.5% ($P = 0.006$). Spermine was protective at a concentration of 0.01 mM, regardless of whether it was administered before or after the exposure of cells to radiation (Fig. 3; $P = 0.013$ and $P = 0.011$, respectively). At a concentration of 0.001 mM, radioprotection by spermine was not as evident for either pre- or post-radiation exposure regimens ($P = 0.257$ and $P = 0.239$, respectively). Both WR-33278 and spermine were effective, regardless of the sequence of administration, in protecting against the induction of *hprt* mutations by radiation (Fig. 4). When administered at a concentration of 0.01 mM, either before or after irradiation, protection by WR-33278 and spermine were highly significant ($P \geq 0.0009$). Even at the lower concentration, both agents conferred protection against the induction of *hprt* mutants ($P \geq 0.04$).

Fig. 3 →

Fig. 4 →

DISCUSSION

It has been suggested that one mechanism underlying the radioprotective effects of aminothiol compounds involves the direct binding of their corresponding disulfide forms to DNA (22). This mechanism would involve the localization of the disulfide moiety to DNA by ionic binding or by the formation of disulfides with protein thiol groups (23,24). A study of this action of disulfides on the processes leading to cellular lethality and mutagenesis is made difficult by the limited ability of these agents to be taken up directly by cells (25). Furthermore, if cells are exposed to the free thiol WR-1065, the intracellular thiol to disulfide ratio is approximately 10:1 (13), making it difficult to effectively study the role of the disulfide in radiation protection.

With the development of the technique of electroporation, it is possible to render cell membranes temporarily permeable to macromolecules such as the disulfide WR-33278. To better investigate the role of WR-33278 in radioprotection and to facilitate a comparison of its radioprotective properties with the polyamine spermine, both agents were introduced under identical conditions into CHO AA8 cells by electroporation. Electroporation by itself has been found to be relatively nontoxic to cells and to be ineffective in producing chromosome aberrations (19). However, it has been reported that mammalian cells can be sensitized to the lethal effects of ionizing radiation by electroporation (26). It has been suggested that this induced radiosensitization is due to changes in cellular tonicity induced by electroporation (26). Our experiments confirmed the radiosensitization effects of electroporation with respect to lethality (Fig. 2*A*) and extended these observations to include the induction of mutations at the *hprt* locus (Fig. 2*B*). The sequence of exposing cells to electroporation and radiation appeared to have no effect on the magnitude of mutants induced per 10^6 surviving cells (Fig. 2*B*). However, irradiation of cells followed 3 h later by electroporation was the more lethal sequence of treatments (Fig. 2*A*). While the reason for

this discrepancy is at present unclear, these findings are consistent with the hypothesis that the mechanisms leading to mutagenesis and lethality are different. Evidence suggesting such a difference comes, in part, from work with the aminothiol WR-1065. Administration of WR-1065 following irradiation is effective in protecting against radiation-induced mutagenesis but not cell lethality (27). It has also been reported that differential protection can be afforded to DNA repair-deficient cell lines against either radiation-induced mutagenesis or radiation-induced cell lethality (28).

The conclusion that the disulfide WR-33278 is ineffective as a radioprotector is based on an analysis of thiol and disulfide contents in WR-1065-treated cells exposed to ionizing radiation (13). Attempts to correlate intracellular WR-1065 contents with levels of cell survival have been highly successful. Disulfide levels, in contrast, remain flat and do not appear to directly correlate with this endpoint. No attempts have been made, however, to correlate amounts of thiol or disulfide moieties bound to DNA with corresponding levels of radiation protection. If the number of potential binding sites on DNA are limited, such as attachment sites to the nuclear matrix, and become saturated as a result of the high concentration of WR-1065 used, a variation in intracellular thiol and disulfide contents as a function of time might not be indicative of their relative importance in conferring radiation protection. Data presented in Fig. 3*A* suggest that cells electroporated with 0.01 mM of WR-33278 30 min prior to exposure to 750 cGy can be protected. Although the levels of cell survival were relatively low, survival protection was enhanced 1.8 fold over control levels and was found to be significant ($P = 0.006$). Consistent with previous reports on aminothiol radioprotection (27,29), no protection against cell lethality was observed when WR-33278 was administered following irradiation.

The electroporation of spermine, 0.01 mM, into cells afforded protection, regardless of whether the polyamine was administered before or following irradiation (PF of 1.4, $P = 0.013$, and PF of 1.4, $P = 0.011$, respectively).

Studies on the possible radioprotective properties of polyamines have focused on assessing radiation response in cells treated with agents such as α-difluoromethylornithine to deplete endogenous polyamine levels (30–32). Under these conditions, there appeared to be an effect on DNA repair (30,31), but it was cell line dependent (32). It was suggested that radiosensitization following the depletion of endogenous polyamines was due to a suppression of cellular recovery processes involved in sublethal and potentially lethal damage repair (32). The addition of exogenous polyamines to these cells gave rise to an increased repair capacity (32), suggesting an important role for polyamines in cellular radioresistance. While both the disulfide WR-33278 and spermine were somewhat similar in their relative abilities to protect cells against the lethal effects of radiation, both agents appeared to be equally effective with respect to their antimutagenic properties. In contrast to the requirement that aminothiols/disulfides be present before radiation exposure to afford protection against lethality, the administration of WR-33278 either before or following irradiation was found to be effective in protecting against radiation-induced mutagenesis at the *hprt* locus (Fig. 4). Spermine also afforded protection regardless of the sequence of treatments of electroporation and radiation used (Fig. 4).

The inability of aminothiols and disulfides to affect cell survival when administered after irradiation suggests that under this condition these agents exerted no effect on the magnitude of repair processes. Rather, the ability of these agents to protect against *hprt* mutation induction under post-radiation exposure conditions suggests that they were affecting the fidelity of repair. Such a model of radiation protection was predicted by Brown in 1967 (22). Through an electrostatic binding of disulfides and/or polyamines to DNA (16,17) not covered

by histones, these regions of chromatin would be stabilized to allow for a decreased DNA replication rate and subsequent enhanced period of time for DNA damage repair processes to be completed. Presumably, the binding of these agents to exposed DNA would also facilitate recombinational repair processes which could lead to lower mutational rates.

Mechanisms of radioprotection attributed to aminothiol compounds include free radical scavenging, chemical repair via donation of hydrogen atoms, auto oxidation, and modification of intracellular enzymatic activities. While each of these processes can account in part for protection against cell killing, only the latter mechanism could account for the antimutagenic effects observed when aminothiols are administered to cells 3 h following radiation exposure (11,27,29). Protection against mutation induction and carcinogenesis are the relevant properties possessed by chemopreventative agents. WR-2721 and its associated metabolites, both the free thiol WR-1065 and the disulfide WR-33278, exhibit these properties (8–11) and thereby offer significant potential for use in the chemoprevention of cancer. The similarities in chemical structure and antimutagenic properties between the aminothiols, their corresponding disulfides, and the polyamines further suggest that they all may share in a similar mechanism of action for chemoprevention. If so, the design of new generations of chemopreventative agents could be facilitated by a better understanding of the role(s) of endogenous polyamines in the process(es) leading to genetic stability and, conversely, mutagenesis.

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FIGURE LEGENDS

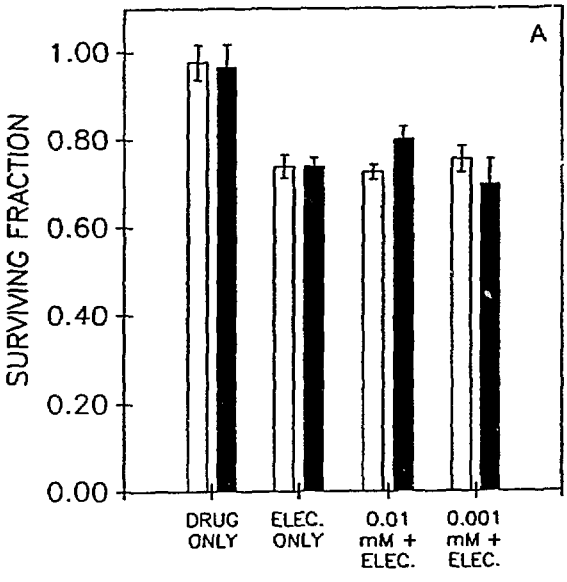
Fig. 1. Effects of WR-33278 (open bar) and spermine (solid bar) on CHO AA8 cell survival (panel A) and mutation induction at the *hprt* locus (panel B). Drug-only bars represent the effects of 0.01 mM WR-33278 or 0.01 mM spermine on these processes. Panel A: compared with its corresponding drug exposure only group, all cell survivals in each of the electroporated groups are significantly reduced (student's two-tailed t test, $P \geq 0.001$). Panel B: compared with its corresponding drug exposure only group, the number of mutants per 10^6 surviving cells in each of the electroporated groups is not significantly different ($P \leq 0.01$). Data presented are from a minimum of 3 replicate experiments. Error bars represent one standard error of the mean.

Fig. 2. Effect of electroporation on radiation-induced cell killing (panel A) and mutagenesis at the *hprt* locus (panel B). Panel A: as compared with cell killing by radiation only, cell survival was significantly reduced by electroporation performed 30 min before ($P = 0.007$) or 3 h after ($P > 0.001$) irradiation. Panel B: mutation induction was significantly enhanced by electroporation performed 30 min before ($P > 0.001$) or 3 h after ($P > 0.001$) irradiation. Experiments were repeated a minimum of 3 times. Error bars represent one standard error of the mean.

Fig. 3. Effect of electroporation with either WR-33278 (open bar) or spermine (solid bar) on the survival of cells irradiated with 750 cGy either 30 min after (panel A) or 3 h before (panel B) electroporation. Panel A: comparing electroporation with no drug 30 min prior to irradiation, electroporation of 0.01 mM WR-33278 or spermine 30 min prior to irradiation significantly protected against cell killing ($P = 0.006$ and $P = 0.013$, respectively). Electroporation of 0.001 mM WR-33278 or spermine was less effective ($P = 0.25$ and $P = 0.02$,

respectively). Panel B: comparing electroporation with no drug 3 h after irradiation, electroporation of WR-33278 did not affect cell survival (0.01 mM, $P = 0.1$; and 0.001 mM, $P = 0.1$). Electroporation of spermine at a concentration of 0.01 mM was more effective ($P = 0.01$) than a concentration of 0.001 mM ($P = 0.23$). All experiments were repeated a minimum of 3 times. Error bars equal one standard error of the mean.

Fig. 4. Effect of electroporation with either WR-33278 (open bar) or spermine (solid bar) on *hprt* mutation induction in cells irradiated with 750 cGy either 30 min after (panel A) or 3 h before (panel B) electroporation. Panel A: comparing electroporation with no drug 30 min prior to irradiation, electroporation of both 0.01 mM and 0.001 mM WR-33278 or spermine were highly effective in protecting against the induction of *hprt* mutants ($P > 0.001$, $P = 0.015$, $P > 0.001$, $P = 0.04$, respectively). Panel B: comparing electroporation with no drug 3 h following irradiation, electroporation of both 0.01 mM and 0.001 mM WR-33278 or spermine were highly effective in protecting against the induction of *hprt* mutants (all P values >0.001). All experiments were repeated a minimum of 3 times. Error bars represent one standard error of the mean.



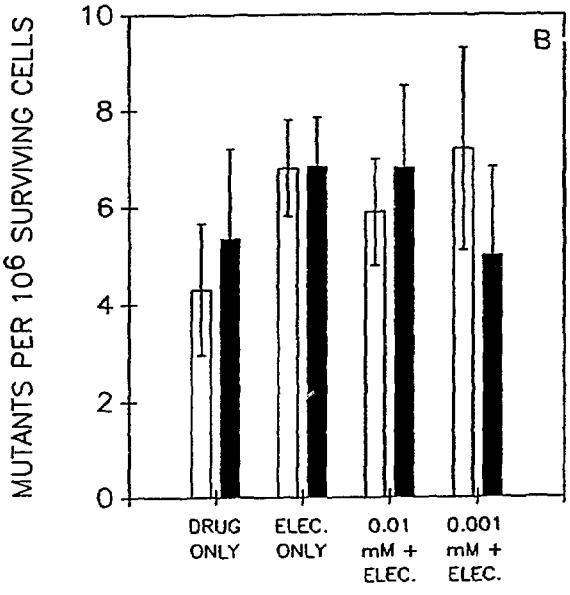
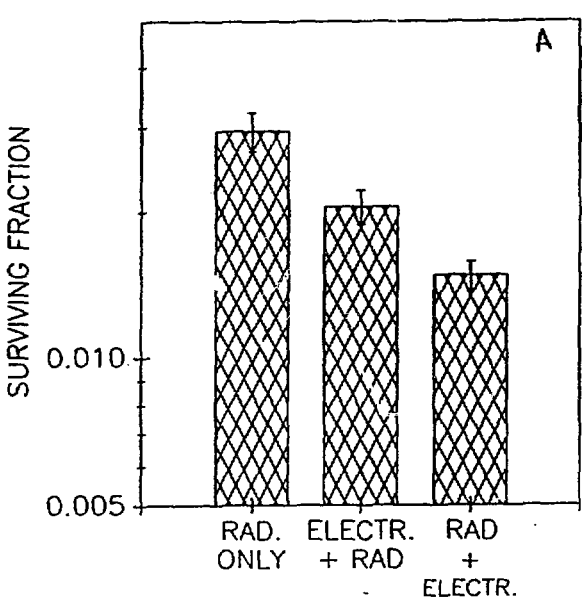


Figure 1. Effects of drug and electroporation on cell survival and mutation (6).



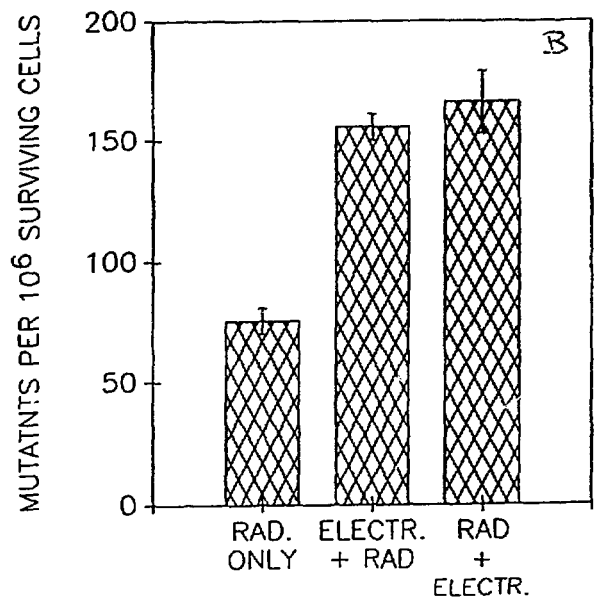


Figure 2. Effect of electroporation on radiation toxicity (A) or mutation induction (B).

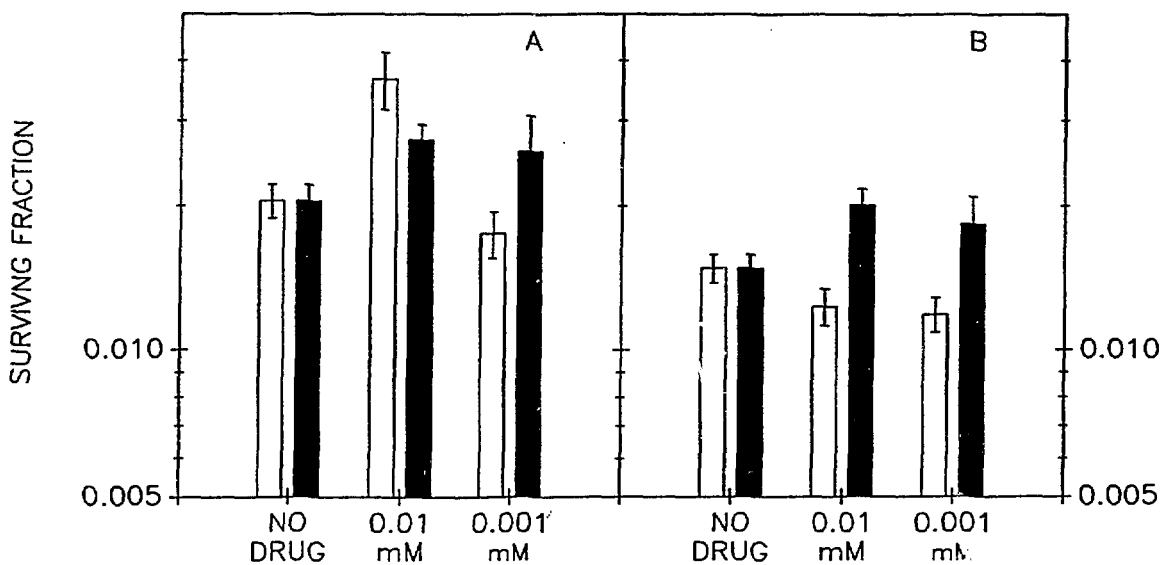

SURVIVNG FRACTION
A
B
0.010
0.005
0.010
0.005
NO DRUG
0.01 mM
0.001 mM
NO DRUG
0.01 mM
0.001 mM

FIGURE 3

E + R

R + E

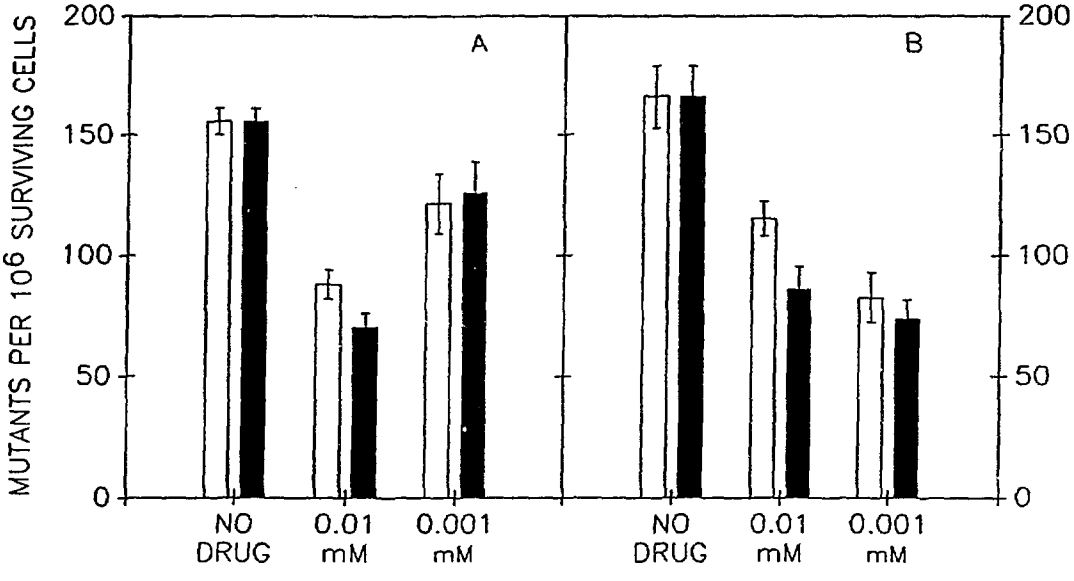


Figure 4. Effect of deoxygenation . . .