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Radiation Protection by Disulfiram: Protection of Membrane and DNA *in vitro* and *in vivo* against γ-Radiation

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Disulfiram/Radioprotector/Comet assay/Lipid peroxidation.

Disufiram (a drug used for the treatment of alcoholism) protected microsomal membranes and plasmid DNA against damages induced by gamma-radiation. The peroxidation of membrane lipids increased linearly with the radiation dose up to 600 Gy, and the presence of disulfiram inhibited membrane lipid peroxidation as assayed by the presence of thiobarbituric acid reacting substances. The reduction of the quantity of the supercoiled (ccc) form of plasmid pBR322 DNA is directly related to the radiation-induced damage, particularly to DNA strand breaks. There was a complete protection of plasmid DNA when exposed to gamma-radiation in the presence of disufiram (0.1 mM) at 300 Gy. This drug also protected deoxyribose against damages caused by hydroxyl radicals produced by the Fenton reaction. The administration of DSF to mice prior to whole-body radiation exposure (4 Gy) resulted in a reduction of peroxidation of membrane lipids in mice liver as well as a decrease in radiation-induced damage to cellular DNA, as assayed by single-cell gel electrophoresis (comet assay). The results thus suggest the possible use of DSF as a radioprotector.

INTRODUCTION

One of the current problems in radiobiological research pertains to the protection of living cells from radiation-induced damage. The search for an effective and non-toxic radioprotector is a major concern in the medical, environmental and space sciences. A variety of compounds with different molecular structures, therapeutic activities and metabolic functions are known to have radioprotective action.[1)]

N,N,N′,N′-Tetraethylthinuram disulfide (Disulfiram, DSF) is wideley used for aversion therapy in alcoholism.[2)] It inhibits aldehyde dehydrogenase, dopamine-beta-oxygenase, microsomal mixed function oxidases and cytochrome P450 enzymes.[3)] DSF is antimutagenic against carcinogens like benz[*a*]pyrene[4)] and Aflatoxin B1.[5)] It is also known to reduce the incidence of chemically induced lung tumors by 32%.[6)] DSF increases the glutathione contents and detoxifying phase II enzymes, like gluthione-S-transferase and UDP-glucuronyl transferase.[7,8)] At low concentration (1 nM) DSF prevents the peroxidation of microsomal phospholipids induced by ADP/Fe^{3+}, indicating its antioxidant capacity.[9)] DSF also inhibits type II lipooxygenases in soyabean,[10)] once again indicating its antioxidant nature. In addition, diethyldithiocarbamate (DDTC), a metabolite of DSF, has been shown to be a potent free-radical scavenger.[11)] In the present studies, an attempt was made to study effect of DSF on radiation-induced lipid peroxidation in microsomal membranes and its possible mechanism of action. In addition, we have studied the protective effect of DSF on radiation-induced DNA damage using a single-cell gel electrophoresis (comet assay) technique.

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Abbreviations: DSF, disulfiram; TBARS, thiobarbituricacid reactive substances.

MATERIALS AND METHODS

Chemical

Disulfiram was purchased from Sigma chemicals (St. Louis, MO, USA), plasmid pBR322 DNA was obtained Bangalore Genei, Bangalore, India and deoxyribose from SRL, India. All other chemicals used were of analytical reagent grade from local manufacturers.

Preparation of rat liver microsomes

Rat liver microsomes were prepared by the method of Shah and Bhattacharya.[12)] Male albino rats of the Wistar strain, weighing 200–220 g, were killed by decapitation. Their livers were quickly removed, cleaned and washed with isolation medium (0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4). A 10% liver homogenate was made in an isolation medium using a Potter-Elvehjem glass homogeniser. The homogenate was centri-

fuged at 9,000 ×*g* for 10 min and the supernatant obtained was spun at 100,000 ×*g* for 1 h to obtain microsomal pellets. Microsomal pellets were washed twice by suspending in 150 mM Tris-HCl, pH 7.0 and re-centrifuged at 100,000 ×*g* for 1 h. Final microsomal pellets were suspended in 0.1 M potassium phosphate buffer, pH 7.4 at 6–8 mg/ml of protein concentration. Microsomes were stored at –20°C and used within 3–4 weeks for lipid peroxidation studies. The amount of protein was estimated by Lowry's method[13] with bovine serum albumin as the standard.

Exposure to gamma radiation

Rat liver microsomes and plasmid DNA were exposed to ^{60}Co-γ rays in a Gamma Cell 220 (AECL, Canada) at a dose rate of 8.0 Gy per min.

Measurement of lipid peroxides as TBARS

Damage to microsomal membranes by gamma radiation was assessed in terms of the lipid peroxidation according to the method of Buege and Aust.[14] After irradiation, the samples were analysed for the presence of thiobarbituric acid reactive substances (TBARS). Briefly, the reaction mixture contained 200–300 µg microsomal membrane protein or 2–3 mg of post nuclear homogenate of 10% liver homogenate, 0.375% thiobarbituric acid, 0.25 N HCl, 15% trichloroacetic acid and 6 mM EDTA. The reaction mixture was incubated at 80°C for 20 min, cooled to ambient temperature and centrifuged at 10,000 ×*g* for 10 min. The amount of TBARS in the supernatant was estimated by measuring the absorption at 532 nm using a Varian DMS 200 spectrophotometer. The lipid peroxidation values are expressed as nmoles of TBARS per mg protein. 1,1,3,3-tetraethoxypropane was used as the standard.

Estimation of DNA

Radiation-induced damage in DNA was determined by irradiating plasmid pBR322 (250–300 ng) at various doses and in the presence and absence of DSF. After irradiation, the DNA was electrophoresed in 1% agarose gel using 0.8 mM Tris borate/2 mM EDTA buffer pH 8.3. (Sambrook *et al.*,[15] R. Rajgopalan *et al.*[16]) The ethidium bromide stained DNA bands were photographed using the Biorad GelDoc system. The photograph was analysed using Syngene software to determine the band intensity.

OH radical scavenging

The antioxidant property of DSF was studied by the method of Elizabeth and Rao.[17] Hydroxyl radicals generated by the Fenton reaction reacted with deoxyribose to form malonyldialdehyde (MDA),[18] which was measured by its reaction with TBA. The reaction mixture consisted 0.1 mM ferric chloride, 0.1 mM ascorbic acid, 0.1 mM of EDTA, 1.0 mM H_2O_2 and 3 mM of deoxyribose in the 20 mM of phosphate buffer pH 7.4. The total reaction volume was 0.5 ml. DSF dissolved in ethanol (1 µl) was added to the reaction mixture in various concentrations and was incubated at 37°C for 1 h. After incubation, 1 ml of the TBA reagent was added. The mixture was then heated in a boiling water bath for 20 min, cooled and A_{532} was determined.

Single cell gel electrophoresis (Comet assay)

The basic alkaline technique described by Gajendiran *et al.*[19] was followed with some modifications. 10 µl of blood from the mice heart was mixed with 80 µl of 0.7% low-melting agarose (Sigma chemicals, St Louis, USA) at 37°C in a microfuge tube and spread on a fully frosted microscopic slide pre-coated with 200 µl of 0.5% normal melting agarose. The slide was covered with a cover slip and left on an ice-cold surface for 2 min after gelling; the cover slip was gently removed. The cells were lysed by dipping the slides into lysing solution (100 mM Na-EDTA, 10 mM Tris, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4°C. The slides were rinsed free of salt and detergent in a buffer (1 mM Na-EDTA, 300 mM NaOH, pH > 13) and subsequently submerged in a horizontal gel-electrophoresis apparatus by adding fresh buffer, and left in the buffer for 20 min to allow the unwinding of the DNA and the expression of alkali labile damage. Then, an electric field was applied (300 mA; 25 mV) for 20 min to draw negatively charged DNA towards the anode. After electrophoresis, the slides were washed twice for 5 min in a neutralising buffer (0.4 M Tris, pH 7.5) and stained with 75 µl of propidium iodide (20 µg/ml). The slides were analysed under a fluorescent microscope with excitation at 530–560 nm coupled with a CCD camera. The comet image was captured and analysed with SGPE pro software. The tail moment (defined as the product of the percent of total fluorescent in the tail and the tail length.) of the 50 cells per slide was recorded. The mean values were calculated. Probability values close to significance were obtained for data groups by employing the one-way ANOVA test. Significance was assumed if $p < 0.05$.

Animals

Male, 8 weeks old Swiss albino mice from our institution's animal house facility weighing 25 g were used. The animals were maintained on standard mouse diet and water *ad libitum* with a 12 h light and 12 h dark cycle.

All experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals for scientific research.

In vivo studies

The animals were divided into four groups (five animals in each group) and treated as follows:

1. Sham-irradiation + 0.3 ml oil po
2. Sham-irradiation + 50 mg/kg DSF, po
3. 4 Gy ^{60}Co-γ-rays + 0.3 ml oil
4. 4 Gy ^{60}Co-γ-rays + 50 mg/Kg DSF, po

Irradiation

Animals were restrained in a ventilated plexibox and the whole body was exposed to ^{60}Co γ-rays at a rate of 0.5 Gy/min using a Junior Telethron teletherphy unit (AEC, Canada). DSF was dissolved in mustard oil and administered per oral (po) to mice 50 mg/kg body weight one hour before irradiation. Animals were irradiated at 4 Gy. Two hours after irradiation, the animals were sacrificed and the livers were excised; the blood was taken for the comet assay. Ten percent liver homogenate was prepared in ice-cold 50 mM potassium phosphate buffer, pH 7.4. The homogenates were centrifuged at 6,000 ×*g* for 10 min at 4°C to get a nuclei-free supernatant. Lipid peroxides were measured following the method of Buege and Aust[14] as described earlier. All of the assays were done in duplicate.

RESULTS

Rat liver microsomes when exposed to γ-rays showed a significant increase in lipid peroxidation, which increased linearly with the radiation dose up to 600 Gy. However, when microsomes were exposed to γ-rays in the presence of DSF (50 μM), there was significant reduction of lipid peroxidation (Fig. 1). It can be seen in Fig. 2 that the radioprotective property of the DSF was dependent on the concentration of DSF. There was a linear increase in the inhibition of TBARS with increasing concentration of DSF up to 50 μM.

Hydroxyl radicals produced by the Fenton reaction can damage deoxyribose to produce malonaldehyde-like substances, which were detected by the TBA reaction.[14,18] Figure 3 shows that DSF inhibited TBARS formation in this reaction, and this suggests that DSF might prevent hydroxyl radical-induced damage to deoxyribose.

Figure 4 and Table 1 show that DSF protected DNA *in vitro* from gamma radiation-induced strand breaks. The reduction of the quantity of the super coiled form (ccc) of plasmid DNA is directly related to the radiation-induced damage—particularly

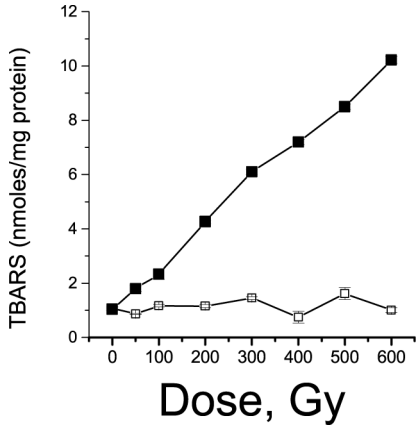

Fig. 1. Changes in lipid peroxidation in microsomal membranes (TBARS nmol/mg protein) at various radiation doses in the presence (open squares) and absence of DSF (solid squares).

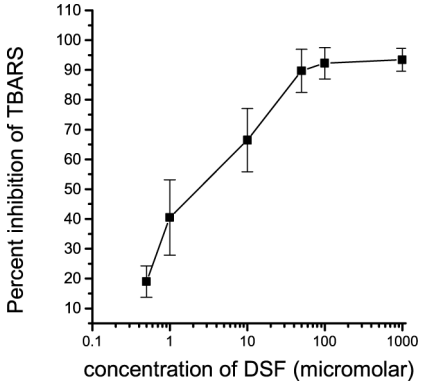

Fig. 2. Changes in lipid peroxidation in microsomal membrane (TBARS nmol/mg protein) exposed to 300 Gy γ-rays at various concentrations of DSF.

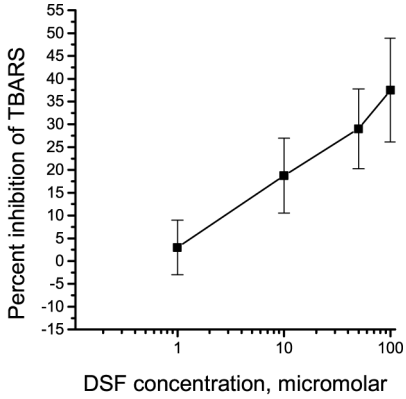

Fig. 3. Changes in TBARS formed during a Fenton-type reaction with deoxyribose at various concentrations of DSF.

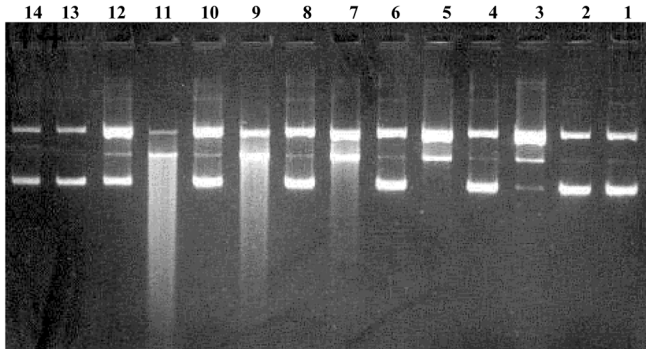

Fig. 4. Agarose gel electrophoresis pattern of pBR322 DNA exposed to various doses of gamma-radiation in the presence and absence of 0.1 mM DSF. The upper and the lower bands depict the open circular (oc) and the supercoiled (ccc) forms respectively. Lanes 1, 2, 13 and 14 are control lanes (without irradiation). Lanes 3, 5, 7, 9, and 11 exposed to radiation without DSF at the gamma radiation doses of 50, 100, 200, 300 and 500 Gy, respectively, without DSF, solvent alone. Lanes 4, 6, 8, 10 and 12 exposed to radiation (at the same doses, respectively) with DSF at a concentration of 0.1 mM.

Table 1. Ability of DSF to protect plasmid pBR322 DNA against γ-radiation induced strand breaks.

Dose of γ-radiation	% ccc form	Protection (%)
0 Gy (Control)	64	–
50 Gy	59	92
100 Gy	56	88
200 Gy	53	83
300 Gy	53	83
500 Gy	36	56

Results of gel photograph (Fig. 4) quantified using Syngene software. For calculation of percentage of super-coiled form in the presence of DSF, the control value (without radiation) was taken as 100%.

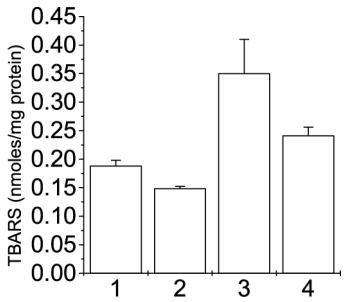

Fig. 5. Changes in *in vivo* lipid peroxidation expressed as TBARS. Mice (n = 5) feed with oil, unirradiated (1), mice feed po DSF 50 mg/kg body weight in oil, unirradiated (2), mice feed with oil, irradiated, 4 Gy (3), mice feed po 50 mg/kg DSF in oil, irradiated, 4 Gy (4). Lipid peroxidation in liver homogenates was estimated as TBARS 2 h after irradiation.

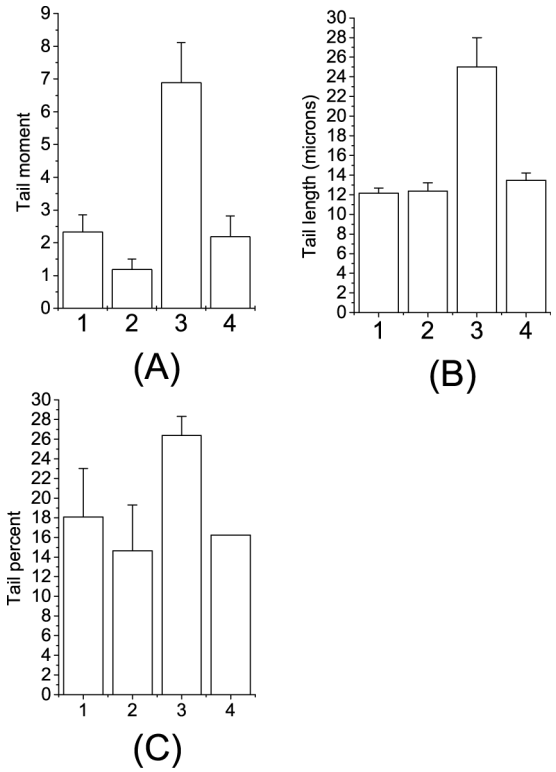

Fig. 6. Effect of DSF on gamma-radiation induced DNA damage in blood lymphocytes, estimated by a comet assay in terms of the tail moment (A), tail length (B), percent DNA in tail (C). 1: control, 2: DSF alone, 3: radiation alone, and 4: DSF fed + radiation.

strand breaks in DNA. As can be seen in the figure, plasmid DNA becomes completely degraded into the small pieces when exposed to higher doses of gamma radiation. There was 92% protection of the plasmid DNA exposed to radiation at 50 Gy in the presence of 0.1 mM DSF.

Figure 5 depicts the extent of peroxide of lipids in the liver of mice exposed to a sub-lethal dose of gamma radiation (4 Gy) after po administration of DSF (50 mg/kg). It can be seen that exposing to 4 Gy gamma radiation resulted an increase in lipid peroxides formed from 0.16 nmol of TBARS/mg protein to 0.38 nmol of TBARS/mg protein. The administration of DSF resulted in a decrease of lipid peroxides formed marginally. However, DSF reduced the lipid peroxides formed by 65% compared to the lipid peroxides formed in the radiation alone group. This also showed *in vivo* that DSF could protect membranes from radiation-induced damage. Figure 6 depicts the results of the comet assay. It can be seen in this figure that whole-body irradiation increased the tail moment, tail length and percent DNA in the tail. The administration of DSF inhibited the increase of these parameters significantly, indicating the protective effects of DSF on radiation-induced DNA damage *in vivo.*

DISCUSSION

Radiation-induced cellular lethality has been ascribed to the formation of aqueous free radicals, which attack vital cellular sites, such as DNA and cell membranes. Radiation-induced peroxidation of membrane lipids, as assessed by TBARS, has been reduced by DSF in a concentration-dependent manner, as can be seen in Fig. 2.

The major damage to DNA inflicted by free radicals is strand breakage.[20] The majority of the free radicals may react with DNA by adding to the double bonds of the bases, forming base radicals. A small percentage of them will react directly with the deoxyribose moiety by abstracting hydrogen atoms, leading to the formation of deoxyribose radicals. Both of these events would lead to strand breaks. This damage when present in sub lethal quantity can produce carcinogenic effects. Our results revealed that DSF effectively protected plasmid DNA against ionising radiation in an *in vitro* system independent of DNA repair and other cellular defense mechanisms. The ability of DSF to scavenge ·OH radicals may contribute to its protective effects against radiation-induced DNA damage. Also, the radiation-induced membrane damage is not due to any of the degradation product(s) of DSF, since even at 500 Gy γ-radiation DSF is found to be stable.[21] Studies on the protection of damage to

deoxyribose from $^{\cdot}OH$ generated by Fenton reaction[18] suggest that DSF is possibly a radioprotector because of free-radical scavenging.

Dithiocarbamates have been reported to show radioprotective activity.[22] However, their radioprotective activities are less than that of mercapto compounds and their derivatives. Several dithioacid dianions exhibit good radioprotection. The radioprotective activity of these non-SH compounds has been suggested to be due to their ability to act as radical-trapping agents through a didative olefin function.[22] Being a non-SH compound, DSF may be offering radiation protection by a similar mechanism. However, further studies are required to unravel the actual mechanism of radioprotection by DSF.

In vivo studies in mice after po administration of DSF suggest that the compound protects tissues from radiation-induced membrane and DNA damage, because there was a significant reduction in radiation-induced lipid peroxidation in whole-body irradiated mice liver. The comet assay result suggests protection to DNA *in vivo* to mice irradiated with 4 Gy γ-rays. The DSF dose used in our studies (50 mg/kg body weight) was far below the LD50 of 1,013 mg/Kg body weight in mice.[23]

DSF has been used as a drug for over 50 years to treat alcoholism.[24]. Thus, in addition to a known application of DSF in aversion therapy in alcoholism, our study shows that DSF has strong radioprotective and antioxidant activities.

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