Materials & methods
3 MATERIALS AND METHODS

3.1 Chemicals

Reduced nicotinamide adenine dinucleotide (NADH), pyrogallol, 1-chloro-2,4-dinitrobenzene (CDNB), RPMI medium (Roswell Park Memorial Institute medium), calcium chloride, calcium ionophore A23187, 2,6-Dichlorophenolindophenol (DCPIP), reduced glutathione (GSH), 1,6 diphenyl-1,3,5-hexatriene (DPH), thiobarbituric acid (TBA), bovine serum albumin (BSA), 5,5-dithio-bis(2-nitrobenzoic acid (DTNB), N-(1-naphthyl)ethylenediamine (NEDD), sulfanilamide, agarose, sodium dodecyl sulfate (SDS), boric acid, acrylamide, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), leupeptin and aproleinin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NF-κB oligonucleotide was obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade.

3.2 Animals

Swiss albino male mice (7-8 weeks old) maintained in the animal house of the University, were used for the present study. Standard feed (Hindustan Lever Ltd.) and water were provided ad libitum. The studies were conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals, Government of India, on the use of animals for scientific research.

3.3 Irradiation of splenocytes with γ-rays

Splenocytes from Swiss Albino mice suspended in RPMI were irradiated in air at room temperature in a gamma chamber (240 TBq ^60^Co Model 4000 A) obtained from Isotope Division, Bhabha Atomic Research Centre (BARC), Mumbai, India. The dose rate used was 0.0575 Gy/sec.

3.4 Preparation of samples

In brief, the animals were killed by cervical dislocation and spleens were removed and washed in normal saline. Single cell suspensions were made in RPMI by
crushing the spleen in between frosted slides. Cells were counted and incubated in RPMI with or without CaCl_2, Diltiazem and calcium ionophore A23187 at 37°C for 30 min before irradiation. They were used as such for assaying peroxidation and fluidity immediately after irradiation; and DNA binding activity of NF-κB, apoptosis and NO* after 30 min, 6 h and 48 h postirradiation respectively. (Time course studies were carried out for all the biological end points examined. The incubation time periods after irradiation were chosen on the basis of significant changes found at the respective time points).

For assay of antioxidant enzymes, the cells were sonicated at a peak to peak amplitude of 18 microns immediately after irradiation and centrifuged thereafter at 16,060g to remove the debris. The supernatant was collected and used for measurement of specific activities of GST, SOD, LDH and DTD.

3.5 Determination of specific activity of superoxide dismutase

Superoxide dismutase was assayed by the method of Marklund and Marklund (1974) by measuring the inhibition of autoxidation of pyrogallol at 420 nm. Assay mixtures (1 ml) contained 0.05 M sodium phosphate buffer (pH-8.0), 0.1 mM EDTA and 0.27 mM pyrogallol. The reaction was initiated by the addition of enzyme sample, which was pretreated with Triton X-100 on ice for 30 min. One unit of enzyme was defined as amount of SOD required to produce half-maximal inhibition of auto-oxidation.

3.6 Determination of specific activity of DT-diaphorase

DT-diaphorase was assayed according to Ernster et al. (1962) by measuring the reduction of 2,6-Dichlorophenolindophenol (DCPIP) at 600 nm with some modification. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM NADH, 40 μM DCPIP, 0.08 % Triton X-100 and enzyme sample in a final volume of 1 ml. The reaction was started at 25°C by addition of NADH. The activity was calculated using an extinction coefficient 21 mM⁻¹ cm⁻¹. One unit of enzyme
activity is defined as amount of enzyme required to reduce one micromole of DCPIP per min

3.7 Determination of specific activity of glutathione-S-transferase

Glutathione-S-transferase (GST) was assayed using the method of Habig et al. (1974), by measuring the formation of GSH-CDNB (1-chloro-2,4-dinitrobenzene) conjugate at 340 nm. Reaction volume (1 ml) contained final concentrations of 0.1 M sodium phosphate buffer (pH 6.5), 1 mM CDNB in ethanol and 1 mM GSH. The reaction was initiated by the addition of enzyme sample. The specific activity was calculated using an extinction coefficient 9.6 mM$^{-1}$cm$^{-1}$ and expressed in terms of micromole of CDNB-GSH conjugate formed/min/mg protein.

3.8 Determination of specific activity of Lactate dehydrogenase

LDH was assayed in the samples spectrophotometrically by measuring disappearance of NADH at 340 nm according to the method of Bergmeyer and Bernt (1974). In brief, the reaction mixture (1ml) contained 0.5 mM pyruvate, 0.1 mM NADH and 50mM phosphate buffer (pH 7.5). The reaction was started by adding enzyme fraction (100 microgram protein/ml). The change in absorbance was measured at 340 nm. The enzyme activity was calculated using an extinction coefficient 6.22 mM cm$^{-1}$ and expressed in units/mg protein. One unit of enzyme activity was defined as amount of enzyme required to oxidize one micromole of NADH per min.

3.9 Determination of membrane fluidity

A 2mM solution of 1,6 diphenyl-1,3,5-hexatriene (DPH) was prepared in tetrahydrofuran and 100 µl of it was added to 100 ml of rapidly stirring Tris-HCl buffer (10 mM, pH 7.4). Treated and irradiated cells were counted and incubated with 2mM DPH for 30 min at room temperature immediately after irradiation. Fluorescence polarization was measured by excitation with vertically polarized monochromatic light at 365 nm and emission intensity detected at 432 nm through a polarizer oriented either parallel or perpendicular to the direction of the polarized
excitation light. The degree of fluorescence polarization was calculated according to Haggerty et al. (1976).

\[ P = \frac{I_{vv} - I_{vh}}{I_{vv} + I_{vh}} \]

Where I is the corrected fluorescence and subscripts v and h indicate values obtained with vertical or horizontal orientation, respectively of the excitation and analyzer polarizer in that order. Fluorescence was monitored on a Shimadzu RF-540 fluorescence spectrophotometer. Because fluidity was inversely related to polarization of the probe, membrane fluidity was expressed as the reciprocal of polarization (1/P).

3.10 Estimation of peroxidative damage

Peroxidative damage was estimated spectrophotometrically, by the thiobarbituric acid (TBARS) method as described by Varshney and Kale (1990) and is expressed in terms of TBARS formed per mg protein. In order to avoid interference due to spontaneous peroxidation of membrane and because sufficient amount of TBARS was formed, the assay was performed immediately after irradiation. In brief, 0.5 ml of cell suspension was mixed with 1.6 ml Tris KCI (0.15 M KCl + 10 mM Tris-HCl, pH 7.4) buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 52 mM TBA (thiobarbituric acid) was added. The tubes were covered with aluminium foil and placed in a water bath for 45 min at 80°C, cooled and centrifuged at room temperature for 10 min at 14000g in REMI-T8 table top centrifuge. The absorbance of the clear supernatant was measured against reference blank of distilled water at 531.8 nm in spectrophotometer (Hitachi 2000).

3.11 Protein determination

Protein concentration was determined by method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

3.12 Determination of non-protein sulphydryl content

Non-protein sulphydryl content was determined by the method described by Moron et al. (1979) in splenocytes immediately postirradiation, using 0.6 mM DTNB
(5,5-dithio-bis(2-nitrobenzoic acid)). The absorbance was read at 412 nm and the sulphydryl content was calculated with the help of standard graph made by using different concentrations of reduced glutathione and expressed in terms of μmol/g tissue. Recently, it has been shown that the sulphydryl content determined by this assay using DTNB, consists mainly of reduced GSH (Snel et al. 1995).

3.13 Determination of nitric oxide levels

5 x 10^6 cells were treated as required and irradiated in RPMI. They were then incubated in a microtitre plate at 37°C in 5% CO2 for 48 hrs. (48 hrs postirradiation was chosen as the time point for the assay after careful time course studies because a good amount of NO* was detectable at this time interval (data not shown)). After that, cells were pelleted and nitric oxide levels were determined in the supernatant by the method of Griess (1879) with some modification. Briefly, to 100 μl of the supernatant, an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylene diamine dihydrochloride(NEDD) in 5% orthophosphoric acid) was added and immediately mixed. After 5 min, 200μl of the above-formed product was transferred to a 96-well flat-bottomed plate and read at 550 nm in a microplate reader. The nitrite content of each sample was evaluated from the standard curve made with sodium nitrite (obtained after linear regression), and was expressed in μM.

3.14 Statistical analysis

Each data point is the mean of observations made from splenocytes from 18 mice (the experiments were carried out thrice with 6 animals in each group each time). The statistical significance of difference between the data pairs was evaluated by analysis of variance (ANOVA) followed by Mann-Whitney U-test.

3.15 Nucleosomal ladder formation assay for apoptosis

Internucleosomal DNA fragmentation was determined by electrophoresis according to the method of Barry and Eastman (1993). Cells were incubated with or without calcium chloride and / or calcium ionophore A23187 in RPMI medium at 37°C for 30 min and irradiated with gamma radiation. In time course studies, a clear
materials and methods

Ladder was observed at 6 h after irradiation compared to earlier time points (data not shown). Therefore, the treated cells were then incubated at 37°C under 5% CO₂ for 6 hrs in RPMI. 2% agarose in Tris-borate EDTA buffer was poured into a horizontal gel support. Once the gel solidified, the section of the gel immediately above the comb was removed by cutting along the top side of the comb with a scalpel and filled with 1% agarose, 2% SDS and 64μg/ml proteinase K. 2 × 10⁶ cells were centrifuged to remove the medium and resuspended in loading dye and RNAse A (1:1 by volume). It was loaded directly into the wells and electrophoresis carried out at 21 V for 16 hrs at room temperature. The gel was visualized under UV illumination after staining with 2μg/ml ethidium bromide.

3.16 Preparation of nuclear extracts

Cells treated as required and irradiated (3 Gy) were incubated in RPMI at 37°C for 30 min (because maximum binding activity was observed at 30 min postirradiation (data not shown)) and subsequently washed twice with PBS, harvested and resuspended in 500μl of buffer containing 20mM HEPES, 1.5 mM MgCl₂, 10 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 10 μg aprotinin, 4 μg leupeptin, 0.2 M PMSF and 1 M DTT. After a 15 min incubation, the cells were centrifuged and resuspended in 50 μl of buffer containing 20 mM HEPES, 1.5 mM MgCl₂, 500 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.1% Triton X-100, 1.0 μg aprotinin, 0.4 μg leupeptin, 0.02 M PMSF and 1 M DTT. After constant agitation for 1 hr at 4°C, nuclear debris was pelleted by centrifugation. The supernatant was stored at −80°C until analysis.

3.17 Electrophoretic mobility shift assay (EMSA) for NF-κB

EMSA was performed with 4μg of nuclear protein in a total volume of 40μl in a buffer containing 20 mM HEPES, 1.5 mM MgCl₂, 50 mM NaCl, 5% glycerol, 1 mM DTT, 0.5 μg poly[d(I-C)], 40 mM EDTA and radiolabelled NF-κB probe at 4°C for 1 hr. The final reaction mixture was analyzed on an 8% polyacrylamide gel with 0.5X Tris borate-EDTA electrophoresis buffer.
3.18 Cell Cultures

Head and neck cancer HTB43, breast carcinoma MDA and lung fibroblasts CCD32 cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were stored in liquid nitrogen with the addition of 5% DMSO to the medium containing 20% fetal calf serum. Stock cultures were grown in DMEM medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 7% CO₂. Normal fibroblasts were used at passage 10-14 for the experiments.

3.19 Drug treatment, irradiation and clonogenic survival assay

Stock cultures of exponentially growing cells were trypsinised into 12.5 cm² culture flasks 60 h prior to treatment. Cell density did not reach confluence during the whole treatment.

The culture medium was changed daily in each flask. Cells assigned for drug treatment alone or in combination with radiation received medium containing equal doses of Diltiazem. For combined treatment, the medium containing the drug was applied prior to or after irradiation depending on experimental conditions. At different time intervals after drug and/or irradiation treatment, cells were plated for survival assays.

For colony forming assays, cells were trypsinised, counted and seeded into six-well plates at a density of 500 cells per well. After incubation for 10 days, cells were fixed with 3.5% formaldehyde and ethanol, stained with Coomassie Blue and Giemsa, and colonies consisting of more than 50 cells were counted.

3.20 Calculation of survival curves

The average plating efficiency of six wells was calculated for each data point. For determination of survival fractions (SF) after treatment with irradiation alone, diltiazem alone or the combination of both agents, the mean plating efficiency of the untreated controls for all days was used as reference.
3.21 Total Protein Extraction

Cells were exposed to 4 Gy to analyze the activation of Caspase-3. At varying time intervals, from 0 to 24 h, after irradiation and DTZ treatment, cells were harvested using Trypsin/EDTA, resuspended in ice-cold medium and centrifuged at 4°C. After washing in ice-cold PBS and centrifugation, cells were lysed using 50 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM MgSO4, 1 mM dithiothreitol, 900 μl / ml complete protease inhibitory cocktail (Boehringer, Germany), 5 mM sodium vanadate and 5 mM sodium fluoride. Five minute incubation on ice was followed by freezing three times in liquid nitrogen and thawing at 37°C. Total protein extracts were determined using a spectrophotometer (Helios Alpha, Unicam, Germany) and stored at –134°C.

3.22 Western blot analysis

50 μg total protein extracts was separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking the membrane in 5 % non-fat dry milk powder in PBS, incubation with primary antibody anti-cleaved Caspase-3 (1: 1000; Cell Signaling technology Inc) was accomplished for 1 h at room temperature. Protein detection employed specific horseradish peroxidase-conjugated secondary antibody anti-rabbit (Santa Cruz, Germany) in combination with the enhanced chemiluminescence detection systems (ECL, Amersham Life Sciences, Germany).