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Radiation Induced Radioresistance – Role of DNA Repair and Mitochondria

Madhu Bala
Radiation Biology Department, Institute of Nuclear Medicine and Allied Sciences
Brig. S K Mazumdar Marg, Delhi,
India

1. Introduction

The bio-positive effects of exposure to small doses of environmental stressors such as radiation, chemicals and mutagens have been reported since long. A number of studies and reviews (Bala & Mathew, 2000; Luckey, 2008; Pandey et al., 2006; Sasaki et al., 2002) document that exposure to small doses of ionizing radiation enhanced the tolerance towards the detrimental effects of lethal doses of ionizing radiation given subsequently. Such phenomenon was observed in prokaryotes as well as in eukaryotes. Some of the laboratory studies with human lymphocytes are summarized in Table 1. The information on beneficial effects of low dose irradiation also poured in from the epidemiological studies (reviewed by Bala & Mathew, 2000; Dasu & Denekamp, 2000; Luckey, 2008). The populations exposed to high background radiation showed long term beneficial effects such as increased life span, enhanced immune system, decreased cancer mortality and cancer risk (Calabrese et al., 2001; Cohen, 1999; Nambi & Soman, 1987, UNSCEAR, 2000). Among the A-bomb survivors from Hiroshima and Nagasaki, those, who received doses lower than 200 mSv, showed no increase in cancer deaths. Further, the population which received doses below 100 mSv, showed decrease in the mortality caused by leukemia in comparison to the age-matched control cohorts (UNSCEAR, 1994).

Often in epidemiological studies the exposure to low levels of radiation was for longer duration, while in laboratory studies the exposure to low level radiation was for a shorter duration (sometimes even a pulse exposure). Nonetheless, beneficial effects were observed in short as well as in prolonged exposures. This strongly suggested that the low dose radiobiological studies could have bearing in diverse and important applications such as radiation protection, risk assessment and radiotherapy. It was, therefore, considered important to initiate investigations for understanding more about the mechanisms of radioprotective effects caused by pre-exposure to low doses of radiation. It was reported that the resistance to lethal doses of ionizing radiation could be induced not only by low doses of radiation but also by variety of agents other than radiation, *viz.* heat, pH, nutrients, UV rays, though, the genes and the molecular pathways affected in these cases differed with the inducing agent. (Bala & Goel 2007; Boreham & Mitchel, 1991; 1994; Boreham et al.,2000). Further, it was believed that the induced resistance to lethal doses of radiation by short term pre-exposure to low doses of radiation was transient in nature and the radiation doses

required to induce beneficial effects varied qualitatively as well as quantitatively from organism to organism. To understand the genetic basis of the phenomenon of low dose induced radioresistance in a comprehensive manner, we chose two different model systems i.e. the microbe *Saccharomyces cerevisiae* and cultured human peripheral blood lymphocytes. The unicellular eukaryote *Saccharomyces cerevisiae* was used to carry out the basic studies and perform the genome wide search for identifying the affected genes. The cultured human peripheral blood lymphocytes were employed to study the role of select genes. In order to minimize the load of mutations, it was considered important to select the smallest possible doses of ionizing radiation for pre-exposure. The term radiation-induced radioresistance (RIR) was introduced (Bala & Goel, 2007) to explain the phenomenon of radioresistance to lethal doses of ionizing radiation, which was (i) specifically induced by a single pre-exposure to sub-lethal doses (causing not more than 10% death of population, $\leq LD_{10}$) of ionizing radiation, and (ii) was transient in nature. The conventional term 'radio-adaptive response' was avoided because the term 'adaptation' in one of the several senses referred to the evolutionary transformations, where new stable behavioral patterns evolved due to prolonged exposure to the environmental stress. This review chapter presents some of our important findings.

References	Important findings
Olivieri *et al.*, 1984	First *in vitro* experiment with human lymphocytes, reduction in chromosomal aberration was greater at higher pre-irradiation dose.
Sanderson & Morley, 1986	Pre-exposure of human lymphocytes to [^{3}H] dThd reduced the number of mutations at *hprt* locus by 1.5 or 3.0 Gy of X-rays.
Shadley & Wolff, 1987	Only stimulated and not G_0 lymphocytes, pre-irradiation with low dose of X-rays showed survival benefit against high doses of X-rays.
Shadley & Wiencke, 1989	The beneficial effects of low dose depended upon total dose, dose rate of pre-irradiated dose but not on dose rate of challenge dose in human lymphocytes.
Boothman *et al.*, 1996	Elevated level of PCNA, cyclin D1, cyclin A in human cell line pre-irradiated with gamma-rays, may play a role in cell cycle regulation and DNA repair.
Carette *et al.*, 2002	Implication of PBP74 in low dose irradiated human tumour cell lines HT29 and MCF-7 with gamma-rays within 30 min after irradiation.
Seo *et al.*, 2006	Role of p27Cip/Kip in the induction of radio-adaptive response in gamma-irradiated RIF cells.

Table 1. Summary of some important studies performed to understand low dose response in human lymphocytes.

2. Materials and methods

The studies were performed sequentially with two different types of cells. Initial studies were executed with the unicellular eukaryotic microbe, *Saccharomyces cerevisiae*, where the focus was to identify the effects of inducing radiation doses and dose rates; the beneficial effects induced in terms of survival, mutagenesis and recombinogenesis; for conducting the genome wide search to identify the affected genes; and reconfirming the role of cell cycle, DNA repair and mitochondrial genes in inducing beneficial effects. The subsequent studies

were conducted with cultured human lymphocytes to investigate some of the key events that were observed in *S. cerevisiae.*

2.1 Studies with *Saccharomyces cerevisiae*

The diploid strain D7 of *Saccharomyces cerevisiae* with genotype a/α: *trp5-12/trp5-27, ilv1-92/ilv1- 92* (Zimmermann *et al.*, 1975) was used because it allowed quick detection of mutants, recombinants and survivors. The strain D7 of *S. cerevisiae* had functional defects in *TRP* gene (heteroallelic) and *ILV* gene (homoallelic) making it auxotrophic for tryptophan and isoleucine. Presence of two different inactive alleles within tryptophan locus (*trp5-12/trp5-27*) caused nutritional requirement for tryptophan which could be recovered by gene conversion to form fully active wild type gene, thereby alleviating the need for tryptophan requirement. The resultant colonies after gene conversion could be scored on synthetic medium lacking tryptophan. The presence of two defective copies of alleles at isoleucine locus *(ilv1-92/ilv1-92)* caused the nutritional requirement of isoleucine which could be corrected by reverse mutation. The resultant colonies could then be scored on the synthetic medium lacking isoleucine. The usefulness of this organism to understand radiation responses and their modification has been demonstrated (Bala & Jain, 1994, Bala & Jain, 1996; Bala & Goel 2004; Bala & Goel 2007).

Cultures of *S. cerevisiae* were grown on yeast extract peptone dextrose medium (YPD; 1% yeast extract powder, 2% peptone, 2% dextrose, HiMedia, India) at 30±1 ^{0}C. The cells were harvested, washed and suspended in phosphate buffer (PB, 67 mM, pH 6.0; 4x10^{7}cells/ml). The cell suspension was cooled to 4 ^{0}C, and irradiated with ^{60}Co- gamma- radiation using Gamma Cell-220 (Atomic energy, Canada; dose rate 0.0078 Gy/s) or Gamma Cell-5000 (BRIT, India; dose rate 1.26 Gy/s). After low dose irradiation, cell suspension was maintained at 30±1 ^{0}C till the subsequent exposure to lethal doses of radiation (LD_{50}, 400 Gy). The survivors, gene convertants and revertants were estimated using defined synthetic complete (DSC), tryptophan omission (TRP-) and isoleucine omission (ILV-) medium, respectively as described (Bala & Goel, 2007). While survivors were expressed as a fraction of unirradiated controls, the gene convertants and revertants were expressed as fraction of CFUs on respective omission medium to the CFUs on DSC medium after the corresponding treatment. The RIR was calculated in terms of percent changes in survivors, convertants and revertants as below:

$$\%\ \text{Change}_{(s,c,r)} = \frac{\text{s / c / r pre} - \text{irradiated} - \left(\text{s / c / r non pre} - \text{irradiated}\right)\ \text{X}\ 100}{\text{s / c / r from non} - \text{pre} - \text{irradiated cells}}$$

Where, s=survivors; c=convertants/10^6 survivors; r=revertants/10^6 survivors (after 400 Gy)

RNeasy Mini Kit and OneStep rt-PCR Kit (Qiagen, Germany) was used to isolate total RNA and carry out rt-PCR respectively (Bala & Goel, 2007). Table 2 enlists the gene specific primers (synthesized from IDT, Coralville). Reverse transcription was at 50 ^{o}C for 30 min, followed by incubation at 95 ^{o}C for 15 min. The amplification was for 25 cycles. The denaturation was at 94 ^{o}C for 45 s; annealing at 60 ^{o}C for 45 s; extension at 72 ^{o}C for 60 s and the final extension was at 72 ^{o}C for 10 min. The PCR products were separated on 1% agarose gel, stained with ethidium bromide and quantified using Lab Works software, version 4.0

(UVP Inc., U.K.). Real-time one-step rt-PCR kit with SYBR green as flourophore (Qiagen, Germany) was used as per manufacturer's protocol to perform quantitative rt-PCR using iCycler (Bio-RAD, US, software version 2.1). The fold changes were determined by calculating the fold change in threshold cycle (Δ Ct').

$$\text{Fold change} = 2^{-(\text{Ct values of control} - \text{Ct value of irradiated sample})}$$

Where Ct: threshold cycle

Gene	Forward primer	Reverse primer
XRS2	AGCAACAATACTGAGAAGG	TGAAATTGGAAATACTCGGA
MRE11	GTCACTCTACCAAGTACTGA	CCATATCACCATATCCAGGAA
RAD50	GGCTTTCATCTCTCAGGA	ATTCCTGGGTGAGGGGAA
SSC1	GTCCCACAAATCGAAGTCAC	GGCATTGTTGCCGTTGTTG
OXI3	GAAGTATCAGGAGGTGGTGAC	TCCCACCACGTAGTAAGTATCG
OGG1	CAGGATGAAAGTGAGCTATGT	CAGATCTATTTTTGCTTCTTTG

Table 2. Primers for genetic studies with *Saccharomyces cerevisiae*

For microarray studies, the labeled cDNA was synthesized from total RNA by using CyScribeTM First-Strand cDNA Labeling Kit (Amersham Biosciences). Either Cy3-dUTP or Cy5-dUTP was incorporated into the cDNA of samples under comparison. The cDNAs were dried in a vacuum trap. Pre-printed DNA microarrays with complete set of 6400 Open Reading Frames (ORFs) of *S. cerevisiae* genome (Microarray Centre of the University Health Network, Toronto, Canada), were used in this study. The slides were first hybridized in pre-hybridization solution (6x SSC, 0.5% SDS, 1% bovine serum albumin) for 1 h and then hybridized overnight with labeled probe at 42 °C in a water bath. Before using as a hybridization probe, the labeled cDNA was re-suspended in 40 µl of hybridization solution (50% Formamide, 6x SSC, 5x Denhardt's, 0.5% SDS, 20 µg of poly(A) and salmon sperm, Invitrogen). For each test, cDNAs from the un-irradiated control and from the stress dose irradiated samples were together hybridized on to one chip. Further, for each test, two different hybridizations were performed by swapping the fluorochromes to cross check the transcriptional changes, if any, due to experimental procedures. At least two DNA microarrays were analyzed for each test condition. The chips were scanned at a resolution of 10 µm and data was analyzed using GenePix Pro 4.0 analysis software (Axon Instruments, Union City, CA).

To study the DNA damage in individual chromosomes by pulsed field gel electrophoresis, the samples were prepared as described earlier (Bala & Jain 1996, Bala & Mathew, 2002). In brief, the cell suspension was washed with PB, centrifuged; pellet was treated with lyticase enzyme and then immobilized in low melting agarose plugs using the mould provided by BioRad USA. The plugs were first treated with LET buffer [0.5 *M* EDTA pH 8.0, 0.01 *M* Tris(hydroxymethyl)-aminomethane pH 7.0, 7.5% β–Mercaptoethanol] for 20 h at 37 ^{o}C. The LET buffer was removed, plugs were washed two times with NDS buffer [0.01 *M* Tris(hydroxymethyl)-aminomethane pH 7.0,7.5% EDTA pH 8.0, 1% n-luaryl sarcosine] . The plugs were then treated with NDS buffer containing 2mg/ml Proteinase K for 20 h at 48 ^{o}C. Sufficient washings were given in EDTA (0.5 *M*, pH 8.0) thereafter. The plugs were stored at 4 ^{o}C before electrophoresis. The pulsed-field gel electrophoreses (PFGE) was for 20 h (60 sec

for first 13 h and 90 sec pulse for next 7h) at 200 V, using CHEF DRII (BioRad, USA), to resolve genomic DNA into a number of chromosomal bands.

2.2 Studies with cultured human Peripheral Blood Mononuclear Cells (PBMC)

Heparinized vacutainers (Griener, Astria) were used to draw 3-5 ml venous blood from healthy, non-smokers, non-alcoholic male donors (age 25-30 years). The blood was layered on the ficoll-histopaque column (Sigma Aldrich Chemicals, USA) and centrifuged at low speed at 26±2°C, the interface between plasma and histopaque comprising PBMCs was collected and washed three times with serum free RPMI-1640 (HiMedia, India). The washed cells were suspended @1×10^6 cells/ml in complete RPMI-1640 containing 10% fetal bovine serum, 100 units/ml penicillin sodium salt, 100 µg/ml streptomycin sulphate, 2 mg/ml sodium bicarbonate. Phytohemagglutinin (PHA, Difco, Hamburg, Germany) was added to stimulate the cells proliferation. The cultures were setup in 96 well flat-bottomed micro titer plates (Tarson, India) at 37°C, 5% CO_2. Each well had 150 µl volume containing 1.5×10^5 cells. The 22-24 hour old cultured PBMCs were irradiated first with low dose of ^{60}Co-γ-radiation (0.07 Gy, using Gamma Cell GC 220, Canada dose rate 0.0078 Gy/s) and then after suitable time interval with lethal dose of ^{60}Co-γ-radiation (5.0 Gy, using Gamma Cell-5000, BRIT, India; dose rate 1.26 Gy/s).

The cell proliferation was quantified using Hoechst 33342. The cells were washed at least three times with saline in microtiter plate, freshly prepared Hoechst 33342 solution in serum free RPMI (10 µg/ml) medium was added and the suspension was incubated at 37°C for 30 min. (Blaheta et. al., 1991). Fluorescence was measured at λ_{ex} 355 nm and λ_{em} 460 nm in fluorescence spectrophotometer (Varion, Australia).

To score the micronuclei, cytochalasin B (Sigma Aldrich Chemicals, USA) was added at 44 hour after initiation of human PBMC culture and the cells were harvested at 72 hour. 1×10^6 cells were washed, the cell pellet was suspended in 200 µl carnoy solution (methanol: acetic acid, 3:1) and incubated at 4°C for 2 hour. This cell suspension was laid on the chilled slides, dried overnight at 26 ± 2°C and stained with hoechst 33342 (10 µg/ml) at 26 ± 2°C for 30 min. in dark. Micronuclei were counted at λ_{ex} 355 nm and λ_{em} 460nm as per criteria described (Fenech, 1993). At least 1000 cells per sample were scored at 1000× magnification under oil immersion.

2.3 Western blotting

Protein extraction and Western blotting was as per procedures standardized in our laboratory (Bala & Goel, 2007). Briefly, $4x10^7$ cells/ ml of *S. cerevisiae* were lysed and treated with 160 ml of 50% trichloroacetic acid (TCA), washed with 1.5 ml of chilled acetone, re-suspended in 100 ml of extraction buffer (4% SDS; 0.16 M Tris-Cl, pH 6.8; 20% Glycerol; 0.38 M b-mercaptoethanol) and heated for 4 min at 95 ^{o}C. For extracting proteins from PBMCs, standard protocol was used. Briefly $5x10^6$ cells were suspended in PB containing protease inhibitors for 1.5 hours at 4 ^{o}C. The cells were ruptured by sonication and soluble proteins were collected after centrifugation in cold. Total soluble proteins were quantified by using Bradford's reagent and resolved by one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN II (BIO-RAD, US). Gels were stained with Coomassie brilliant blue R-250. Electro-blotting was on nitrocellulose membrane

(Millipore) and treatment with primary and secondary antibody was as described earlier (Bala & Goel, 2007).

2.4 Statistical analysis

Each experiment based on CFUs assay, had three replicates and was repeated at least three times. The data was presented as the average of three experiments $\pm$ S.D. For estimating differential gene expression, DNA damage and protein expression, the data was analyzed using paired t-test. For cell survival, mutagenesis and recombinogenesis the data was analyzed using two-way ANOVA. $P \leq 0.05$ was considered significant.

3. Results and discussion

3.1 Studies with *Saccharomyces cerevisiae*

3.1.1 RIR inducing doses, survival and mutagenesis

Systematic study with cultures grown to different phases (mid-log phase, late log phase and stationary phase) showed that the stationary phase cultures did not show any RIR. Mid-log phase cultures showed 25% increase, while late log phase cultures showed only 12% increase in survivors in comparison to the non-pre-irradiated cultures. This comparison was made at pre-irradiation dose 20 Gy (LD_{10}), challenge dose 400 Gy (LD_{50}) and the time interval between stress dose irradiation and challenge dose irradiation 4.5 h (Sharma & Bala, 2002). This was in agreement with earlier reports (Cai & Liu, 1990), where mitogen stimulated human lymphocyte cultures showed far better radio-adaptive response than the resting cells. The RIR, since was maximum with mid–log phase cells, further studies were planned with the mid-log phase cells. Pre-treatment with three different doses of ^{60}Co-gamma-ray viz. 4, 10 and 20 Gy ($\leq LD_{10}$) showed that RIR increased with increase in the pre-irradiation dose. In comparison to non-pre-irradiated controls, the 4 Gy pre-irradiated samples showed maximum 13% increase in survivors, 10 Gy pre-irradiated samples showed maximum 27% increase in survivors and 20 Gy pre-irradiated samples showed maximum 32% increase in survivors after lethal irradiation (400 Gy). The time of maximal increase in survivors was delayed at higher stress doses and was approximately 10 h after irradiation at 20 Gy, 6 h after irradiation at 4 or 10 Gy (Figure 1). However, there was no linear correlation between the pre-irradiation ^{60}Co-gamma-ray dose and increase in survival due to RIR. These studies suggested that priming of cells with small radiation doses may induce some signaling events which may lead to RIR. The pre-irradiation (stress) dose (20 Gy), thereafter, was delivered at two different dose rates i.e. 0.0078 Gy/s and 1.26 Gy/s to the mid-log phase cells. It was observed that in comparison to non-pre-irradiated cultures, the cultures pre-irradiated (20Gy) at lower dose rate (0.0078 Gy/s) and lethally irradiated with 400 Gy, showed a maximum of 32% increase in survivors while cultures pre-irradiated at higher dose rate (1.26 Gy/s) showed a maximum of 25% increase in survivors after lethal irradiation. Further, in comparison to non-pre-irradiated cultures, the pre-irradiated cultures showed decrease in gene convertants and revertants when irradiated with lethal dose (400 Gy, Dwivedi et al., 2001). The dose rate also impacted the mutations and gene conversion quantum and time kinetics. Pre-irradiation dose (20 Gy), delivered at lower dose rate decreased the gene conversions and mutations for a longer time period in comparison to the same dose delivered at higher dose rate (Figure 1).

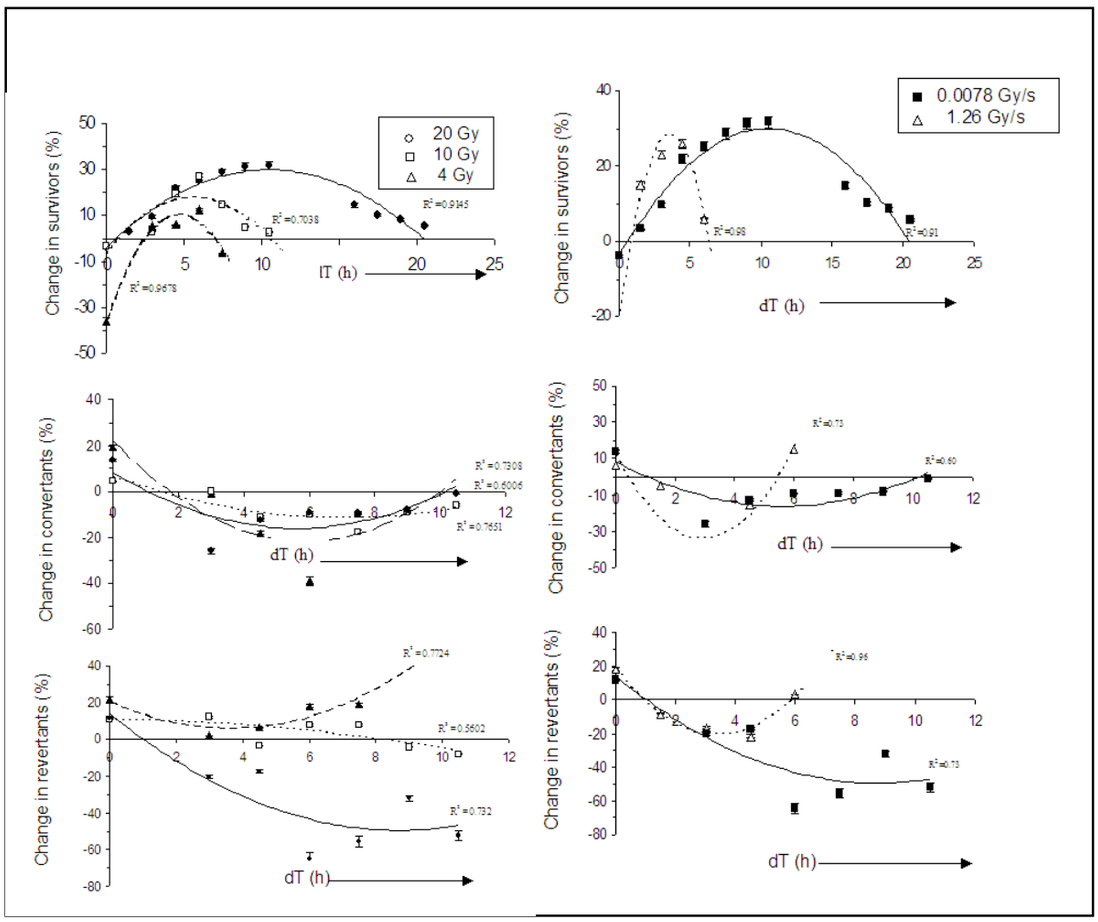

Fig. 1. Effect of different pre-irradiation doses (4,10, 20 Gy) and of dose rates of stress dose (20 Gy) on RIR in *Saccharomyces cerevisiae.* dT (h): duration in hours between pre-irradiation and lethal irradiation (400 Gy). The values are average ± S.D. of three experiments. (From Dwivedi et al., 2008).

There are reports to show that there is difference in the nature and quantum of DNA damage by stress doses delivered at different dose rates (Chaubey et al., 2006). The higher RIR (survival) by stress doses delivered at lower dose rate as compared to the same stress dose delivered at higher dose rate suggested that nature of damage generated by stress dose, is an important determinant of induction of protective mechanisms.

3.1.2 Alteration in gene expression after irradiation with low dose of ^{60}Co-gamma-radiation - whole genome analysis

As many as 110 open reading frames (ORFs) displayed more than 2 fold increase in transcription at 4.5 h after the low dose irradiation (20 Gy) and some of the annotated once are listed in Table 3.

The functional groups of the up-regulated genes were DNA damage, repair, synthesis, energy generation, metabolism and stress response. Besides this, many transcripts with

unknown function (not listed in Table 3), were also up-regulated. Some genes such as IRE1, HSP12 were down-regulated 4.5 h after irradiation (20 Gy) (Table 4). Sahara et al., 2002 reported that Hsp12p might play a role in protein binding in yeast. The Ire1p and Hac1p participate in the "unfolded protein response" (UPR) pathway. It is predicted that in our study the UPR pathway was down regulated. Other genes that were down regulated were DDR48, MSN2. Further studies are planned to understand the role of these genes and the pathways in which they participate to induced RIR.

ORF	Gene	Function
Stress response		
	HSP78	Heat shock protein
YOL053C	*DDR2*	DNA damage stress response
YDL229W	*SSB1*	cytosolic HSP70
YER103W	*SSA4*	cytosolic HSP70
Signal Transduction		
YKL161C	*MLP1*	Serine-threonine kinase
YKL168C	*KKQ8*	Serine threonine protein kinase
YOL016C	*CMK2*	Ca/calmodulin dependent kinase
YHR030C	*SLT2*	MAP kinase pathway
Energy and Metabolism		
YDL022W	*GPD1*	Glycerol-3P-dehydrogenase
YJL155C	*FBP26*	Fructose/mannose metabolism
YPL088W	*ALD4*	Putative aryl dehydrogenase
YOR374W	*HOR2*	Mitochondrial Aldehyde
YER062C	*PRB1*	dehydrogenase
YEL060C	*GTT1*	DL-glycerol-P-phosphase
YIR038C	*CIT1*	Protein degradation
YNR001C	*PDC1*	Glutathione transferase
YLR044C	*ADH1*	TCA cycle; citrate synthase
YOL086C	*RHR2*	Glycolysis, pyruate decarboxylase
YIL053W	*FAS2*	Alcohol dehydrogenase
YPL231W	*COX4*	Glycerol metabolism
YGL187C	*STF2*	Fatty acid biosynthesis
YGR008C	*TPS2*	Cytochrome C Oxidase
YDR074W	*PHO5*	ATPase stabilizing factor
YBR093C	*NCA3*	Glucose, fructose metabolism
YJL116C		Phosphate metabolism
YLR327C		Mitochondrion biogenesis
		Organelle biogenesis
Transcription and Translation		
YKR062W	*TFA2*	RNA pol II transcription factor
YNL301C	*RP28B*	Constituent of ribosome
YNL178W	*RPS3*	Constituent of ribosome
YFR031CA	*RPL5B*	Component of ribosome
YDL083C	*RPS16B*	Constituent of ribosome
YGL031C	*RPL30A*	Component of ribosome
YOR204W	*DED1*	mRNA processing helicase
YDR088C	*SLU7*	mRNA processing
YLR325C	*RPL38*	ribosomal protein L38
YDR280W	*RRP45*	3'->5' exoribonuclease

ORF	Gene	Function
DNA damage, repair, synthesis		
YER164W	*CHD1*	chromodomain-helicase-DNA-binding (CHD) family
YML061C	*PIF1*	DNA helicase
YER095W	*RAD51*	Recombinase
YER018C	*SPC25*	spindle pole body component
YDR201W	*SPC19*	spindle pole body component
YNL222W	*SSU72*	nuclear protein
YNL206C	unknown	similar to SSRP proteins (DNA structure-specific)
YMR137C	*SNM1 /PSO2*	required for inter-strand crosslink repair
YNL250W	*RAD50*	DNA binding protein
YOL090W	*MSH2*	MutS homolog; mismatch repair
YCR014C	*POL4*	DNA polymerase IV
YER088C	*DOT6*	Nuclear protein with Myb DNA-binding domain
YBL019W	*APN2*	exonuclease III homolog (AP endonuclease)
YCR088W	*ABP1*	actin binding protein
YDR545W	*YRF1*	Y' helicase (subtelomerically-encoded)
YJL065C	unknown	similar to DNA polymerase epsilon subunit c
YNL088W	*TOP2*	DNA topoisomerase II
YJR021C	*REC107*	ds break formation complex subunit
YKL113C	*RAD27*	ssDNA endonuclease
YGL163C	*RAD54*	DNA-dependent ATPase
YDR225W	*HTA1*	Transcription; Chromatin binding
YGLO37C	*PNC1*	Cell aging
Cell cycle regulatory		
YER059W	*PCL6*	cyclin (Pho85p)
YLL065W	*GIN11*	growth inhibitor
YDR285W	*ZIP1*	synaptonemal complex protein
YAL063C	*FLO9*	*cell wall protein*
YBR211C	*AME1*	microtubule associated
YNL289W	*PCL1*	G1/S cyclin
YPR120C	*CLB5*	G1/S cyclin
YDR217C	*RAD9*	Cell cycle, DNA damage
YFL029C	*CAK1*	Cdk-activating kinase

Table 3. Up regulated (> 2.0 folds) transcripts, 4.5 h after the irradiation (20 Gy). Categorization of ORFs into functional groups is based on SGD Library.

Gene	Function
HSP26	Heat shock protein
GPD1	Glycerol-3-phosphate dehydrogenase (NAD^+),cytoplasmic
HSP12	Heat Shock protein
HSP30	Heat Shock protein
SSA1	Cytosolic HSP70
YGP1	Secreted glycoproteins
ECM32	DNA dependent ATPase/DNA helicase B
ATH1	Acid trehalase, vacuolar
ARN1	Ferrichrome-type siderophore transporter
DDR48	Heat Shock Protein
MSN2	Stress-responsive regulatory protein

Table 4. Some important genes down regulated (≥1.5 folds) after the 20 Gy irradiation

3.1.3 Confirmation of stress dose induced time dependent changes in selected transcripts as well as associated genes

3.1.3.1 The MRX complex

Significant over expression of genes from the DNA damage, response, repair complex, prompted us to perform real time quantitative PCR for the *MRX* complex (*MRE11, RAD50* and *XRS2)* of which *RAD50* is an essential gene. The β-actin gene, though considered as a house keeping gene, showed differences in the stress dose irradiated cultures in comparison to the non-pre irradiated cultures, suggesting that β-actin gene could not be used as a house keeping gene. The experimental data was, therefore, compared with reference to the un-irradiated control at the corresponding time. The results obtained from the real time quantitative rt-PCR (Figure 2) confirmed the significant increase in *RAD50* transcripts at 4.5 h in stress dose irradiated cultures and supported the information obtained by microarray (Table 3).

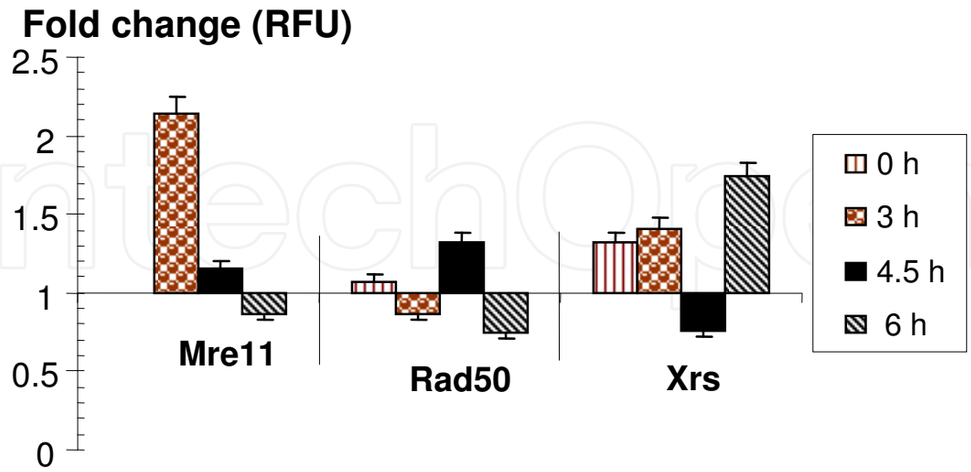

Fig. 2. Effect of stress dose irradiation on relative time dependent changes in gene expression of MRE11, RAD50 and XRS2 as studied by Real Time- reverse transcription PCR; the value of untreated control was assigned as one. RFU: relative fluorescence units (from Dwivedi et al., 2008)

The *RAD50/MRE11* complex possesses single-strand endonuclease activity and ATP-dependent double-strand-specific exonuclease activity. Rad50 provides ATP-dependent control of mre11 by unwinding and/or repositioning DNA ends into the MRE11 active site. The rt-PCR studies showed that in non-irradiated controls, there was significant increase in *MRE11* transcript level from 0 h to 4.5 h. In comparison to the non-irradiated controls, the stress dose irradiated samples showed significantly higher level of *MRE11* transcripts at 3 h time interval after irradiation. Further, in comparison to non-irradiated controls, the stress dose irradiated samples showed significantly higher levels of *XRS2* up to 3 h and reduced levels at 4.5 h after irradiation. The Mre11 complex influences diverse functions in the DNA damage response. The complex comprises the globular DNA-binding domain and the Rad50 hook domain, which are linked by a long and extended Rad50 coiled-coil domain. Recently it is reported that functions of *MRE11* complex are integrated by the coiled coils of Rad50 (Hohl et al., 2011). *MRE11* is reportedly involved in DNA double-strand break repair and possesses single-strand endonuclease activity and double-strand-specific 3'-5' exonuclease activity. Its role in meiotic DSB processing is also reported (Smolka et al., 2007).

3.1.3.2 The heat shock proteins

The western blotting studies with members of *HSP70* family showed that in untreated controls, level of *Kar2 p* did not increase significantly from 0 h to 4.5 h. In comparison to non-irradiated controls, the stress dose irradiated samples showed significantly higher *Kar2 p* level at 3 and 4.5 h. The *Ssa1p* transcript level did not change in the untreated control from 0 h till 4.5 h. In stress dose irradiated samples, the *Ssa1p* level increased up to 3 h but decreased significantly at 4.5 h, in comparison to the non-irradiated control (Bala & Dwivedi 2005). By microarray technique also the *SSA1* level were found to be lower in the stress dose irradiated samples as compared to the untreated control at 4.5 h (Table 4). In stress dose irradiated cultures, the *Ssa2p* level was significantly higher than the non-irradiated control at 0h and 3 h (Figure 3).

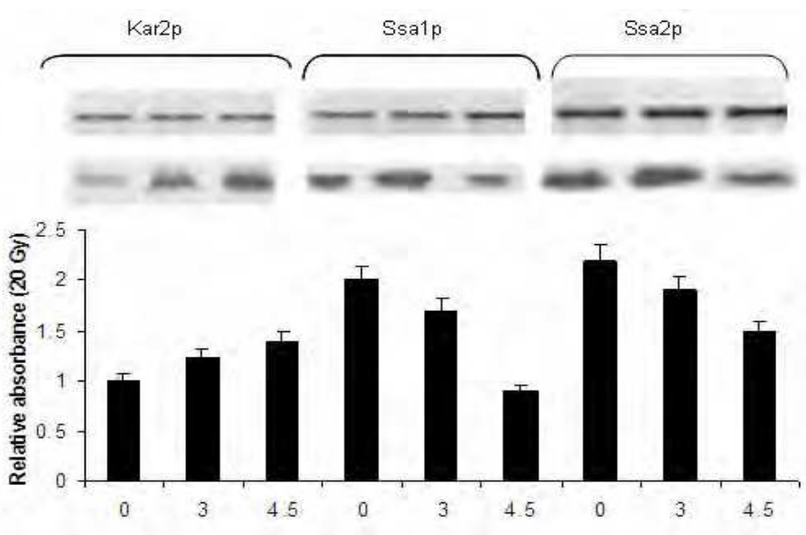

Fig. 3. The effect of low dose irradiation (20 Gy) on expression of Kar2p, Ssa1p and Ssa2p. The top strip of membrane blot shows the protein expression in unirradiated controls.

3.1.3.3 The mitochondrial genes

Saccharomyces cerevisiae is an excellent eukaryotic model system to study DNA repair mechanisms because DNA repair pathways are highly conserved between human and yeast. Furthermore, yeast and human mitochondria resemble each other in structure and function. Mitochondria are the major sites of energy (ATP) production in the cell. Mitochondria also perform many other cellular functions, such as respiration and heme, lipid, amino acid and nucleotide biosynthesis. Mitochondria also maintain the intracellular homeostasis of inorganic ions and initiate programmed cell death. Mitochondria are the major source of endogenous reactive oxygen species (ROS) in cells as they contain the electron transport chain that reduces oxygen to water by addition of electrons during oxidative phosphorylation. The rt-PCR studies with the mitochondrial genes (*SSC1* gene coding for mtHsp70, *OXI3* gene coding for COX1 respiratory component of complex-IV and *OGG1*gene) showed that the expression of *OXI3* was more than unirradiated controls up to 6 h and that of *SSC1* only at 2 and 10 hours after irradiation (20 Gy, Figure 4a,b). The expression of OGG1 was increased up to 2 hour only, after irradiation [(20 Gy), data not shown]. The mitochondrial genome of eukaryotic cells is extremely susceptible to damage due to constant exposure to significant amounts of reactive oxygen species (ROS) produced endogenously by mitochondria as a by-product of oxidative phosphorylation (Gupta et al., 2005). It is known that inactivation of *OGG1* in yeast leads to spontaneous mutations in the mitochondrial genome. Our analysis revealed that irradiation with low doses of gamma radiation enhanced the expression of expression of OGG1 at 2 h only.

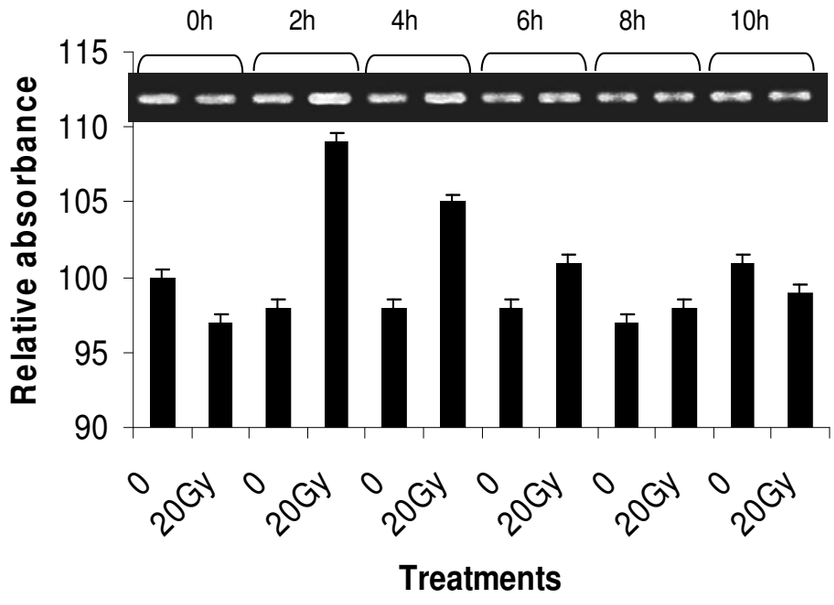

Fig. 4a. Expression of *OXI3* gene after low dose pre-irradiation at different time intervals in *Saccharomyces cerevisiae* (From Arya et al., 2006).

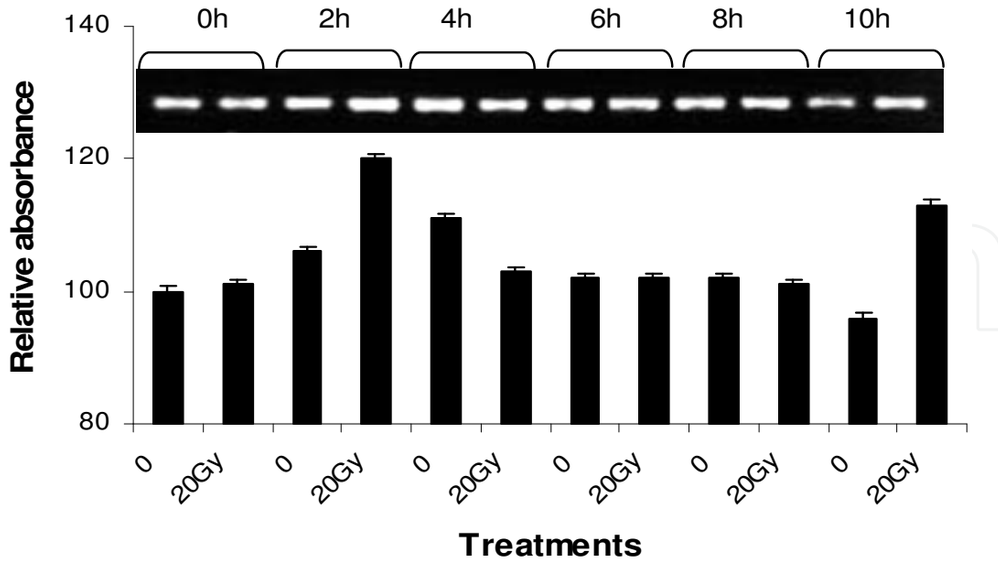

Fig. 4b. Expression of *SSC1* (mt-HSP70) gene after low dose pre-irradiation at different time intervals in *Saccharomyces cerevisiae* (From Arya et al., 2006)

Maintenance of mitochondrial DNA (mtDNA) is essential for ensuring respiratory competence. *MGM101* was identified as a gene essential for mtDNA maintenance in *S. cerevisiae*. The MGM101p binds the DNA. The MGM101 function exclusively in the repair of DNA contained in the mitochondrial organelle, and is predicted to participate in base excision and/or nucleotide excision repair pathways. *Saccharomyces cerevisiae* contain 3 different Hsp70s i.e. *SSC1*, *SSQ1*, and *SSC3*. Amongst these, *SSC1* is the most abundant constitutively expressed multifunctional Hsp70 and is essential for the viability of yeast cells. It plays a critical role in protein translocation across the mitochondrial inner membrane and folding of almost all pre-proteins targeted to the mitochondrial matrix compartment, thus maintaining protein homeostasis in mitochondria. For proper translocation function, SSC1p moves to the translocation Tim23-channel as a core component of "import motor complex" via the peripheral membrane protein, Tim44. Besides translocation function, SSC1p plays a crucial role in folding of proteins that are imported into the mitochondrial matrix The mitochondrial genome of eukaryotic cells is extremely susceptible to damage due to constant exposure to significant amounts of ROSs produced endogenously by mitochondria as a by-product of oxidative phosphorylation. It is known that inactivation of OGG1 in yeast leads to spontaneous mutations in the mitochondrial genome. Our analysis revealed that irradiation with low doses of gamma radiation enhanced the expression of OGG1 after 2 h. The inactivation of human OGG1 is known to induce both the spontaneous and induced mutations in the mitochondrial genome.

3.1.4 Sequencing of mitochondrial genes

The amplified product of two of the mitochondrial genes *COX1* and *SSC1* were sequenced using commercial services. No significant change was observed in DNA sequence of both

these genes after low dose (20 Gy) irradiation (Figure 5a and 5b). This suggested that a RIR inducing doses were not inducing any mutations in the gene products.

Fig. 5a. Sequencing of COX1 gene PCR product by forward primer (top chart) and reverse primer (bottom chart). Irradiation with low dose (20 Gy) did not induce changes in DNA sequence.

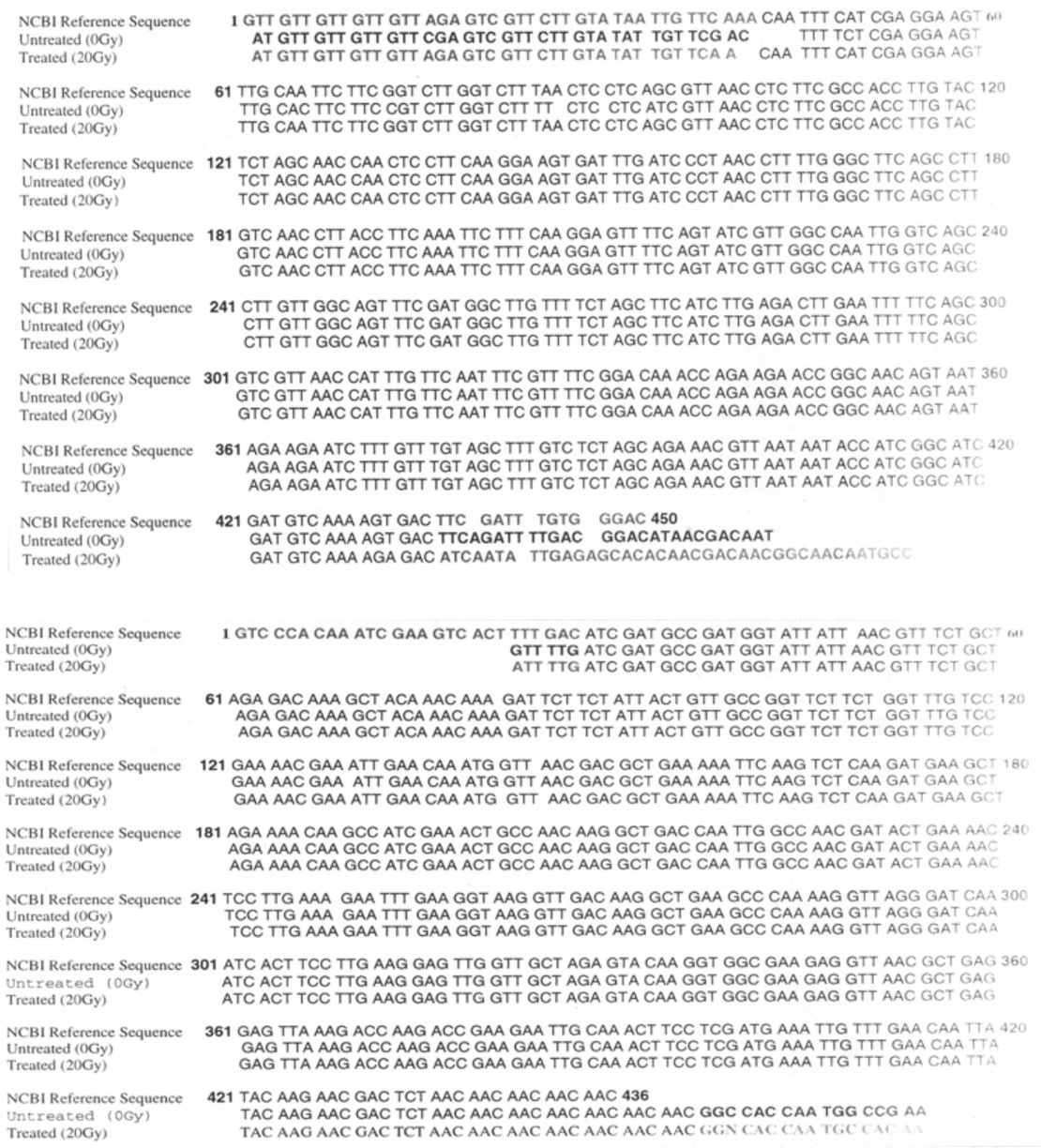

Fig. 5b. Sequencing of SSC1 (mt HSP70) gene PCR product by forward primer (top chart) and reverse primer (bottom chrt). Irradiation with low dose (20 Gy) did not induce changes in DNA sequence.

3.1.5 Chromosomal DNA damage and repair in *S. cerevisiae* cells showing RIR

Study of chromosomal DNA damage was considered important because of its role in low dose induced responses (Bala & Dwivedi 2005; Collis et al., 2004). *S. cerevisiae* has small genome divided into sixteen chromosomes of sizes ranging from 240 to 2200 kb, which can be easily resolved into discrete bands by pulsed-field gel electrophoresis (PFGE). In our study plan, PFGE could resolve the genomic DNA into several bands (Figure 6 a,b,

Lane 1). No significant change in the fluorescence intensity or mobility of bands could be recorded after low dose irradiation (20 Gy) (Lane 2). However, in comparison to untreated controls (lane1), in samples irradiated with 200 Gy, there was observable decrease in the fluorescence intensity of high molecular weight bands and increase in the smear intensity along the lanes (Figure 6, lane 3). This suggested that 20 Gy was too small a radiation dose to cause sufficiently large number of double strand breaks to be detected by PFGE. Although, presence of other types of DNA damage viz. base damage, DNA cross-links or single strand breaks as predicted by ionizing radiation at this dose could not be ruled out. The increase in the smear along the lanes in 200 Gy irradiated samples was due to settling down of broken DNA fragments along the lanes. This was similar to our earlier observations with X-irradiated (Bala & Jain, 1996) and ^{60}Co-γ–irradiated yeast cells (Bala & Mathew, 2002) and indicated that radiation dose 200 Gy could cause DNA double strand breaks immediately after irradiation. The samples pre-irradiated with 20 Gy, incubated in PBG for 2 h and then irradiated with 200 Gy showed greater DNA bands intensities in higher molecular weight region (Lane 4) as compared to non-pre-irradiated but 200 Gy irradiated samples (Lane 3). This suggested that cells which were pre-irradiated with 20 Gy and maintained in PBG for 2 h prior to lethal dose (200 Gy) irradiation, suffered considerably lower DNA damage as compared to the lethally-irradiated

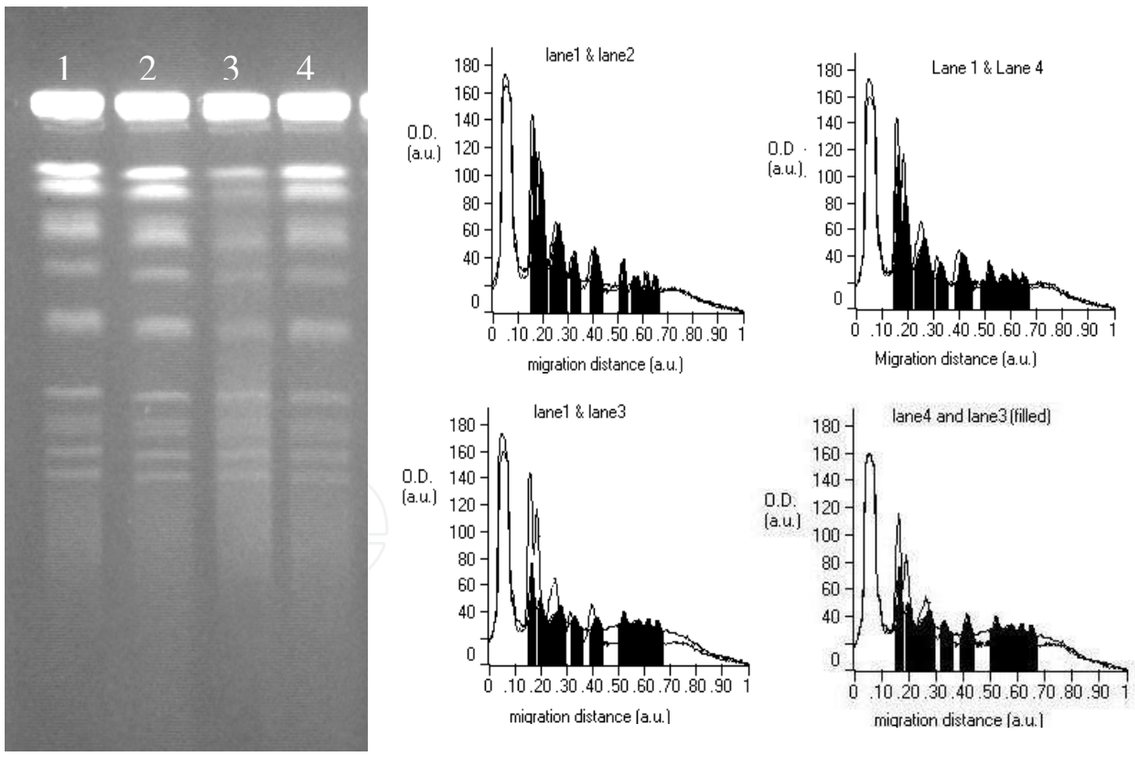

Fig. 6. (Gel picture and corresponding densitometry): The pre-irradiation with low dose (20 Gy) reduces chromosomal DNA damage induced by lethal doses of ^{60}Co-γ-radiation (200 Gy) as studied by pulsed-field gel electrophoresis. Lane1: untreated control;
Lane 2: ^{60}Co-γ-ray (20 Gy); Lane 3: ^{60}Co-γ-ray (200 Gy); Lane 4: ^{60}Co-γ-ray (20 Gy) + incubation in PBG for 2 h + ^{60}Co-γ-ray (200 Gy).

cells (lane 3). During analysis of pulsed-field gels throughout this study, the intensity changes in the individual bands in the lower molecular weight region were not given much importance because their intensities were influenced by the intensities of DNA fragments settling down as smears in the lower molecular weight regions and this has been shown to create errors in data analysis in our earlier studies (Bala & Jain 1996, Bala & Mathew 2002). **I**nduction of gene transcription or protein expression has been reported after low dose irradiation (Franco et al., 2005). Our studies showed that low dose radiation enhanced DNA repair ability and produced protective proteins to minimize the indirect damaging effects of subsequent high dose radiation.

3.2 Studies with human PBMCs

The phenomenon of radioadaptive response has been reported in human lymphocytes in various studies (Table 1). The advantages of the lymphocytes as a model to understand the low dose ionizing radiation response is due to their radiosensitivity. More over, the lymphocytes are found in circulating peripheral blood and therefore, can be easily obtained from peripheral venous blood. These cells involved in cell mediated immunity as well as humoral immunity and cell proliferation and their radiosensitivity is similar to that of proliferating cells of the hematopoetic tissue.

3.2.1 RIR in human PBMCs - cell proliferation, micronuclei formation

Hoechst binds with DNA and an increase in fluorescence is observed with the increase in cell number. This assay was used to determine the effects of low doses of ^{60}Co-gamma-radiation on proliferation of PBMCs. The cells pre-irradiated with low dose of ^{60}Co-gamma-rays (0.07 Gy) and 4-5 hours later irradiated with high dose ^{60}Co-gamma-rays (5 Gy, LD 50) showed significantly higher cell proliferation (RIR), in comparison to non-pre-irradiated but lethally irradiated (5 Gy) cells. The RIR, however, was much less before 4h and after 5h of time interval between low dose and high dose exposure (Bala et al., 2002). The maximum increase was observed if ^{60}Co-gamma-rays (5 Gy, LD 50) were given 5 h after the low dose (0.7 Gy) irradiation. In comparison to the non-pre-irradiated controls, the pre-irradiated cells showed decrease in micronuclei frequency and the decrease varied between the donors from 23.4% - 31.8% (Table 5, Bala et al., 2002).

Donors	Micronuclei per 1000 binucleated cells at doses (Gy)				% decrease
	0	0.07	5.0	0.07+5.0	
I	13±0.33	15±1.02	245±11.08	167±8.92*	31.8±2.29
II	18±0.89	16±1.26	166±9.67	89±6.65*	23.4±2.14
X	12±0.65	14±0.88	298±15.38	206±8.77*	30.8±2.47

Table 5. Micronucei (MN) in human PBMCs stained with hoechst 33342 and total 1000 cells were scored per sample at 400 × magnification in fluorescence microscope. % decrease in MN = ($MN_{low\ dose+high\ dose}$ -$MN_{low\ dose}$) X 100/$MN_{high\ dose;}$ * indicates significant ($p<0.05$) change with respect to 5.0 Gy irradiated controls (from Bala et al., 2002).

3.2.2 Effect of RIR on MRN complex proteins in human PBMCs

The *MRE11/RAD50/NBN* (*MRN*) complex in humans comprises genes, which are homologous/analogous the to *MRX* complex in *S. cerevisiae.* The *MRN* complex is

involved in double-strand break (DSB) repair, DNA recombination and cell cycle checkpoint control (Carson et al., 2003). The complex participates in single-strand endonuclease activity and double-strand-specific 3'-5' exonuclease activity. The protein expression of Mre11p in low dose irradiated cells was enhanced (about 1.5 times) as compared to non pre-irradiated cell after 5.0 hours of irradiation. This was similar to the enhanced the expression of *MRE11* in *S. cerevisiae* (Table 3, Figure 2). *NBS1* or *p95* is another component of the *MRN* complex, which has a role in the recruitment of the *MRN* complex to double strand break sites for DNA repair. *NBS1* plays a critical role in the cellular response to DNA damage and the maintenance of chromosome integrity. *NBS1* modulate the DNA damage signal sensing by recruiting PI3/PI4-kinase family members ATM, ATR, and probably DNA-PKcs to the DNA damage sites and activating their functions (Frappart 2005; Stiff et al., 2005). It can also recruit *MRE11* and *RAD50* to the proximity of DSBs by an interaction with the histone H2AX. *NBS1* also functions in telomere length maintenance by generating the 3' overhang which serves as a primer for telomerase dependent telomere elongation. The Nbs1p levels in low dose irradiated cells were significantly reduced (nearly 2 times) as compared to non-pre-irradiated cell after 5.0 hours of irradiation. It is not clear why the protein levels were reduced. *NBS1,* since, is inducible gene, time dependent studies are now planned to understand the role of *NBS1* in RIR. After 5 hour of low dose exposure Rad50p level was similar as in unirradiated cells (Figure 7). *RAD50* is required to bind DNA ends and hold them in close proximity This could facilitate searches for short or long regions of sequence homology in the recombining DNA templates, and may also stimulate the activity of DNA ligases and/or restrict the nuclease activity of *MRE11A* to prevent nucleolytic degradation past a given point (Jager et al., 2001, Waltes et al., 2009). In our study, the levels of RAD50p did not alter 5 hour after low dose irradiation in comparison to the untreated controls.

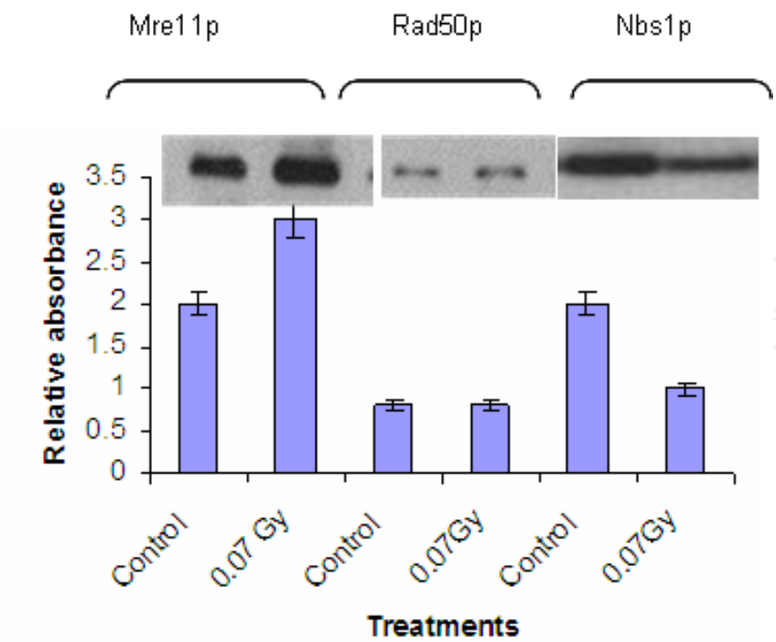

Fig. 7. Change in expression of Mre11p, Nbs1p and Rad50p in human PBMCs 5 hour after irradiation with low dose (0.07 Gy) of ^{60}Co-γ-rays. Results were mean ±SD.

4. Summary and conclusion

Exposure to low dose radiation could be of significance in clinical evaluation of risk assessment, radiotherapy and radiation protection. Although a number of mechanisms such as enhanced DNA repair, alterations in stress proteins, immuno-modulation, and antioxidant defense system have been proposed to contribute to beneficial effects of low dose exposure, the understanding about the mechanisms inadequate. This is primarily because the reports are scattered, and among the available reports there is variability of dose response, the model systems as well as the experimental design followed in different laboratories. Our studies with a uniform experimental design on two different model systems viz. *Saccharomyces cerevisiae* and human PBMCs, has clearly demonstrated that irradiation with lower doses of ionizing radiation has a beneficial effect on the organism. Moreover, the effect of lower dose of radiation can not be predicted simply by extrapolating the effect of higher doses. The term 'Radiation induced radioresistance' or 'RIR' was suggested in our studies to refer a phenomenon where a single small dose radiation exposure could lead to better tolerance to the subsequently given lethal doses of radiation, and the effect was transient. Although, the RIR caused by low dose irradiation appears to be a complex interplay of many genes, this study shows that the genes of *MRX/MRN* complex, *HSP* family and also mitochondrial gene have a confirmed role in phenomenon leading to RIR. *KAR2* is an integral component of unfolded protein response (UPR) pathway. Up regulation of *KAR2* negatively regulated UPR pathway (Kimata et al., 2003), and, therefore may have caused accumulation of unfolded cytosolic proteins. If this is true then, after low dose irradiation, up regulation of *KAR2* helped the accumulation of proteins that might have unfolded due to radiation stress. The accumulation of unfolded proteins may have been responsible for increased levels of *SSA1 /SSA2 /SSA4* similar to that observed in *S. cerevisiae* cells after heat shock (Stone & Craig 1990). Also, it was observed the mitochondrial genes for maintaining the functional integrity of mitochondria; as well as to counter the reactive oxygen species that may have been produced because of oxidative stress produced after irradiation, were up-regulated. Further, the absence of mutation in representative sequences, decrease in revertants as well as tryptophan prototrophs, decrease in the micronuclei frequency together with enhanced levels of error-free DNA repair, strongly suggested that priming with low doses imparted transient radio-resistance to the cells culminating in the survival benefits via error-free mechanisms.

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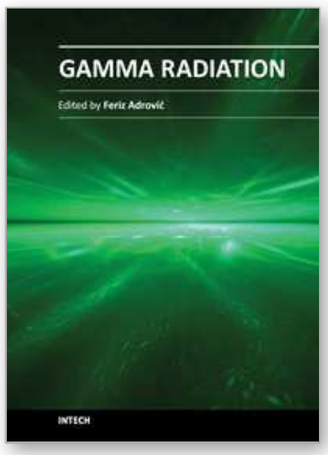

Gamma Radiation

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This book brings new research insights on the properties and behavior of gamma radiation, studies from a wide range of options of gamma radiation applications in Nuclear Physics, industrial processes, Environmental Science, Radiation Biology, Radiation Chemistry, Agriculture and Forestry, sterilization, food industry, as well as the review of both advantages and problems that are present in these applications. The book is primarily intended for scientific workers who have contacts with gamma radiation, such as staff working in nuclear power plants, manufacturing industries and civil engineers, medical equipment manufacturers, oncologists, radiation therapists, dental professionals, universities and the military, as well as those who intend to enter the world of applications and problems of gamma radiation. Because of the global importance of gamma radiation, the content of this book will be interesting for the wider audience as well.

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University Campus STeP Ri
Slavka Krautzeka 83/A
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Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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