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Detection methodologies for radiation hormesis and radioadaptive response

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Abstract : Both radiation hormesis (RH) and radio-adaptive response occur at a very low dose (10^{-4} Gy to 10^{-2} Gy). For detection of such a low dose there are physical, chemical, biochemical, biopolymeric, cellular and genetic radiation-induced radiobiological end points. The present article represents quite a large number of such techniques and methodologies such as thermo-luminescence (TLD), chemical dosimeters, biopolymeric dosimeters, polymerase chain reaction (PCR), autoradiography, flow cytometry, confocal microscopy, colony survival assay, electron spin resonance (ESR), chemiluminescence, oxidative DNA damage, markers for oxidative stress, micronucleus assay, DNA double strand break assay (DSB), commet assay, fluorescence life time analysis, oxidative stress by fluorimetric assay, gamma H2A X foci technique etc. The principle of each technique has been mentioned.

The benefits of these techniques as applied to different health and disease aspects of the society have been discussed.

Keywords : Radiation hormesis, radio-adaptive response, principles of different methodologies, benefits to the society.

Introduction

Radiation hormesis[1-3] and radioadaptive response[4-6] are known to the scientific community for more than two decades. These have acquired significant importance in recent years.

As radiation hormesis occurs at a very low dose of radiation of the order of 10^{-4} Gy to 10^{-2} Gy, accordingly the instrument should be sensitive enough to detect this dose. There are various dosimeters available for this purpose. But these should satisfy the following criteria : (a) Linearity in dose-response relationship. (b) The energy range in which the response is linear. (c) Response should be independent of dose rate. (d) Temperature co-efficient of variation in response should be known. (e) Small amount of change in response in the chemical or biological system should be possible to be measured. (f) Response to noise ratio should be as large as possible. (g) Reproducibility should be established with a maximum variation of $\pm 5\%$. (h) Stability in response, especially for biological systems should be mentioned.

Some of the hormetic expressions like total weight of a particular part of the organism are easily detectable. But heavy metal induced or toxic chemical induced modulation in the functional aspect of organisms need especial techniques for their detection. Similarly biological endpoint detection needs special techniques. Measurement of radiation hormesis consists of two parts : (i) Measurement of low dose. (ii) Measurement of low dose induced changes in chemical, biochemical or biological expressions.

Measurement of low dose :

(a) Thermoluminescence dosimeter :

A thermoluminescent dosimeter namely CaF_2 : Dy has been mostly used for low dose measurement. The lowest detection limit of it has been reported to be 0.05 mSv and the energy response range is from 8.5 to 100 keV. This dosimeter has been used to detect background radiation dose in Tanzania for 7 years (1993-1999). The background environmental radiation (cosmic, terrestrial) along with that of phosphate rock, waste tailings and con-

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trol soil were measured. For this purpose two sets of TLDs were employed in each station and dose rates were determined by the following equation :

$$R = \frac{1}{N_t}\sum_i (g_i - m)/S_i \times FK - D$$

where S_i = mean value of relative sensitivity of individual dosimeters, N = number of dosimeters, R = dose rate (nGy per hour), t = time in hours between TLD placement and withdrawal at station, g_i = gross readout of individual dosimeters from the stations, m = mean readout of background dosimeters, F = calibration factor for TLD reader during monitoring period and K = correction factor for fading and D = correction for transit dose (nGy per hour)[6].

(b) Low level chemical dosimeters :

Radiation causes reactions which create at least one, initially absent chemical substance to be long lived enough to allow determination either of its quantity or change in the properties of the original system. For this the basic requirements are : (i) Chemical change caused by radiation should be proportional to the absorbed dose and the chemical change should not be below the sensitivity of analytical methods. (ii) The chemical change should be independent of dose rate, linear energy transfer of the radiation, temperature and air. The dosimeter should be stable both before and after irradiation, particularly against influence of air and light.

Unless the radiation-induced reaction occurring in the dosimeter is a chain reaction, the concentration of the product will generally be less than 1 μmole per Gy. This concentration is either close to or lower than the limit of most analytical techniques. Mostly these dosimeters are tissue-equivalent as the percentage composition of the elements are close to that of a tissue.

For radiation hormesis (RH) and radio-adaptive response (RAR) the chemical dosimeters should have the following characteristics : (i) dose range should be low i.e. 0.001 to 10 Gy, (ii) dose rate range should be 0.40 to 2 Gy/mins, (iii) should be tissue-equivalent, (iv) G-value should be known and (v) should be stable.

There are a large number of chemical dosimeters which satisfy the above criteria. Some of these have been developed by us along with others. Table below gives the necessary salient features for a few dosimeters that are usable for RH and RAR (Table 1).

(c) Biopolymers as dosimeters :

Ionising radiation penetrates biological cell membrane, nuclear membrane and nucleolar membrane and finally damages the chromosomes. The chromosomes are constituted of nucleoproteins which are again constituted of deoxyribonucleic acids, ribonucleic acids and proteins. Radiation-induced deformations in the structure of DNA and proteins, when found proportional to dose, can be used as a dosimetric system. The advantage here is that biopolymers are constituents of tissues and cells. So the absorbed dose received by biopolymers is a close approximation to that of tissues. Here also the same dosimetric characteristics are required as in a chemical dosimeter. But radiation-induced changes are irreversible here and stability of dose induced changes can be kept

Table 1

Dosimeter	Chemical changes measured and method of measurement	Dose range (Gy)	Yield	Stability	Ref.
Irradiated Fricke + ^{59}Fe ions	Ion exchange partition	0.01–0.40	-	Not good	7, 8
FBX	Fe^{2+} to Fe^{3+}, spectrophotometry	0.001–30	$G(Fe^{3+}) = 65$	Good	9–17
Terephthalic acid	Fluorescence	0.03–8	-	Good	18
Salicylic acid	Fluorescence	0.005–0.05	G (product) = 0.10	Not good	19, 20

reproducible at 7-8 °C. As compared to chemical dosimeters the biopolymeric dosimeters respond at a higher dose and less stable. Given below a few methodologies with salient features for some of the biopolymers.

Deoxyribonucleic acid : For Calf Thymus DNA (conc. 4.17×10^{-9} ML^{-1}) *in vitro* in solution showed the following dose-response characteristics in 0.1 *M* phosphate buffer, pH 7.0. Linearity in absorbance intensity at 207 nm and 259 nm up to a dose of 50 Gy was observed. It did not show any variation in the position of absorption maximum. Percentage hyperchromicity at 207 nm and 259 nm were 20% and 18% respectively for a dose of 50 Gy at a dose rate of 1.5 Gy/min. The average $A_{207\ nm}$/Gy and $A_{259\ nm}$/Gy values were found to be 0.0031 and 0.0022 respectively (Upadhyay 2005). These are the slopes of these two lines. Percentage hyperchromicity for DNA (conc. 30 μg per ml) at an ionic strength of 2.00, pH 6.05-7.94 was found to be 1.00 percent for a dose of 10 Gy[21].

H_1 Histone : H_1 histone (conc. 1.163×10^{-6} ML^{-1}) in 0.1 *M* phosphate buffer, pH 7.0 showed a linear response at $A_{202\ nm}$ and $A_{270\ nm}$ as a function of dose up to 70 Gy at a dose rate of 1.50 Gy/min. Percentage hyperchromicity at 202 nm and 270 nm were 11% and 12.6% respectively. The average $A_{202\ nm}$/Gy and $A_{270\ nm}$/Gy values were found to be 0.0024 and 0.0042 respectively. These are the slopes of two lines. The fluorescence intensity of H_1 histone (conc. 3.326×10^{-6} ML^{-1}) in 10^{-3} *M* phosphate buffer at pH 7.0 decreased consistently with dose at a dose rate of 0.009 Gy/s up to 100 Gy by 60% ($Ex_{280\ nm}$, $Em_{338\ nm}$)[22] (Table 2).

DNA-H_1 Histone complex[22] : The $A_{206\ nm}$ and $A_{260\ nm}$ values increased linearly with dose up to 50 Gy in 0.1 *M* phosphate buffer for DNA : H_1 complex. Similarly $A_{226\ nm}$ values and $A_{260\ nm}$ values also increased linearly with dose up to 50 Gy in 0.15 *M* SSC buffer for DNA : H_1 complex. Percentage hyperchromicity was 18% at 206 nm and 260 nm in 0.1 *M* phosphate buffer but that in 0.15 *M* SSC buffer the values were 20% at 226 nm and 18% at 260 nm for the same dose. The average $A_{206\ nm}$/Gy and $A_{260\ nm}$/Gy in 0.1 *M* phosphate buffer were found to be 0.0042 and 0.0013 respectively. Similarly average $A_{226\ nm}$/Gy and $A_{260\ nm}$/Gy in 0.15 *M* SSC buffer were found to be 0.0024 and 0.0020 respectively. These are the slopes of these four lines. In 0.1 *M* phosphate buffer the concentration ratio for DNA : H_1 histone complex was 4.17×10^{-9} ML^{-1} : 1.163×10^{-6} ML^{-1}. In 0.15 *M* SSC buffer the concentration ratio of DNA : H_1 histone complex was 2.085×10^{-9} : 9.304×10^{-7} ML^{-1}.

Table 2

Dose (Gy)	Fluorescent intensity count (slit width 0.5 nm; $Ex_{280\ nm}$ $Em_{338\ nm}$)
Unirradiated	460 ± 10
25	350 ± 20
50	290 ± 10
75	240 ± 10
100	190 ± 10

n = 3

DNA-H_3 Histone complex : When the complex of DNA : H_3 (50 : 50 μg/ml) in 0.9% sodium chloride was irradiated up to 50 Gy at a dose rate of 0.0106 Gy/s (pH range 6.90 to 6.64), no change in absorption maximum was found. Absorption intensities decreased consistently with dose at 260 nm and 206 nm reaching a plateau at 40-50 Gy[23]. Effect of radiation dose on the absorption properties and pH of DNA : H_3 complex (50 : 50 μg/ml), solvent 0.9% sodium chloride at a dose rate of 0.0106 Gy/s is given below (Table 3).

Table 3

Dose (Gy)	λ_{max_1} (nm)	λ_{max_2} (nm)	$A\lambda_{max_1}$	$A\lambda_{max_2}$	pH
Unirr.	206	260	1.88	1.48	6.90
10	206	260	1.73	1.46	6.86
20	206	260	1.68	1.39	6.82
30	206	260	1.67	1.36	6.70
40	206	260	1.70	1.26	6.64
50	206	260	1.70	1.26	6.64

H_4 Histone[24] : In 0.01 *N* hydrochloric acid, pH range 3.92 to 4.44, H_4 histone (concentration 100 μg/ml) showed a linear decrease in absorbance intensity at $A_{210\ nm}$ and $A_{220\ nm}$ from unirradiated solution to irradiated solution up to a dose of 50 Gy at a dose rate of 1.12 Gy/s. Again H_4 histone (concentration 50 μg/ml) in 0.9% sodium chloride, pH 6.80 (maintained by 0.15 *M* phosphate buffer) when complexed with Folin Ciocalteau reagent were irradiated at a dose rate of 1.12 Gy/s the $A_{660\ nm}$ values decreased in a linear manner with dose up to 80 Gy. The

minimum dose detected was 20 Gy.

DNA : H_4 Complex[24] : There was a linear increase in H_4 histone depletion from DNA : H_4 histone complex (DNA : H_4 25 : 25 μg/ml) in 0.9% sodium chloride, pH 6.80 from 20 to 70 Gy at a dose rate of 1.12 Gy/s (Table 4).

Table 4. Depletion of H_4 histone from DNA-H_4 complex as a function of dose

Dose (Gy)	A_{660nm}	H_4 histone depleted (μg/ml)
Unirr.	0.003	0.00
20	0.010	3.00
30	0.015	4.00
40	0.020	6.00
50	0.030	8.50
70	0.040	11.00

(d) Polymerase chain reaction (PCR) :

It is a method of amplifying a target sequence of DNA. PCR provides a sensitive, selective and extremely rapid means of amplifying a desired sequence of DNA. Specificity is based on the use of two oligo-nucleotide primers that hybridize to complementary sequences on opposite strands of DNA and flank the target sequence.

The DNA sample is first heated to separate the two strands, the premises are allowed to bind to the DNA and each strand is copied by a DNA polymerase, starting at the primer site. The two DNA strands each serve as a template for the synthesis of new DNA from the two primers. Repeated cycles of heat denaturation annealing of the primes to their complementary sequences and extension of annealed primers with DNA polymerase result in the exponential amplification of DNA segments of defined length. DNA sequences as short as 50–100 base pair and as long as 2.5 kilo base pair can be amplified. The applications of PCR are in forensic medicine, detection of infectious agents, detection of prenatal genetic diagnosis, establishment of precise tissue types for transplants, to study evolution, to study gene modifications, point mutations, deletions, insertions and rearrangements of DNA etc.[25,26].

(e) Autoradiography :

The biological sample containing the radioisotope is placed in close contact with a sheet or film of photographic emulsion. Rays emitted by the radioisotope enter the photographic emulsion and expose it in a manner similar to visible light. After a period of several days, the film is developed and the location of the radioisotope in the original sample determined from exposure spots on the film. The method is most often used with histological sections to determine the precise location of a leveled compound in a tissue or in a cell and may be applied in the light microscope or electron microscope level. It is used for identifying zones containing leveled compounds[27].

(d) Flow cytometry :

Lasers are commonly used as light sources to excite fluorochromes that are coupled to anti-bodies or other proteins. The light emitted from the laser excited fluorochrome is then passed to a detector (PMTS) where it is converted to electronic signals where these are processed to yield the data points for the measured objects. Maximum number of laser sources are four. Hence to simultaneously measure a large number of fluorochromes multiple dyes have to be excited by the same laser. Each fluorochrome should emit at a wavelength distinct from that of any other fluorochrome, so that they must have distinct emission spectra. Most fluorochromes have emission spectra that overlap at least partially with those of other fluorochromes. These partial overlaps are corrected in flow cytometry in two ways :

(i) By blocking out unwanted light before data collection by installing filters that selectively pass light of a narrow band of light onto a detector (bandpass filters).

(ii) Through a process of measurement correction called compensation. It mathematically removes spillover signals emitted from fluorochromes other than those to be measured.

Compensation is applied to flow cytometric measurements before or after data acquisition. The following needs scrutiny for getting maximum benefit from flow cytometry : (a) Spectral characteristics of fluorochromes. (b) Chemical characteristics of fluorochromes. (c) Relative brightness of a fluorochrome. (d) Power of laser and its wavelength for obtaining maximum signal intensity. (e) Suitable photomultiplier tubes with minimum noise and high signal intensity. (f) Appropriate standardization and data analysis.

Recently multicolour flow cytometry is being used to

measure simultaneously up to 12 colours and scatter parameters. The standard dyes used were : Fluorescein thiocyanate, Phycoerythrin and Allophycocyanin. Special features of these flow cytometers are : (i) Identification of cells and cell subsets based on surface receptor expression profiles. (ii) Identification of smaller cell subsets and different functional cell subpopulations has become possible[28-31].

(g) Confocal microscopy :

A confocal microscope does not improve resolution in horizontal plane beyond that of a standard optical microscope. It offers the capability to optically section a specimen into slices 700 nm thick and thus minimizes blurring from structures located at a distance from the plane of focus. The elimination of blur increases image contrast and consequently makes possible the full utilization of the spatial resolution offered by the optical components of the microscope. A fluorescence confocal microscope is used to record fluorescence intensity as a function of position in two dimensional (2D) or three dimensional (3D) space. It has a spatial resolution of few hundred nanometers and it can visualize even single molecules. In biological samples several parameters may need to be recorded. Hence the requirements are that : (a) There should be several intensity signals in 3D space versus time. (b) Different fluorescence life times, intensities and wavelengths of scattered lights should be measurable.

From these physical parameters the biologically relevant values can be estimated. These are : (a) Local concentration of a probe. This can then be translated to the concentration of a target molecule (e.g. DNA or protein). (b) Properties of molecular environment like membrane fluidity, diffusion of molecules, fluorescent proteins, bound and unbound pool of an active protein in a living cell etc.

The best resolution achievable in the plane of a periodic object with a objective of high numerical aperture (NA) using light of wavelength λ is :

$$d = a.\lambda\ (n.\sin\alpha)^{-1}$$

where d = the smallest distance between two points that can be visually resolved, a = a constant, dependent on the adopted criterion of resolution (a = 0.5 in theory), λ = the wavelength of light that forms the image, n = refractive index of medium between specimen and objective lens, α = half the solid angle between the outermost rays entering the lens.

In confocal microscopy only a few (up to 20) photons are collected per bright pixel in one scan. The detector has a limited quantum efficiency and the electronics may have its own fluctuations. The following processes are needed essentially :

(i) Calibration of fluorescence and measurement of fluorescence intensity ratio.

(ii) Maintaining both detection channels in register.

(iii) Accounting for different photobleaching rates.

(iv) Proper subtraction of background signals and autofluorescence.

(v) Avoiding artifacts caused by phototoxicity and light induced reactions.

In practice for one image collection at maximum resolution requires 5–30 s.

Limitations of confocal microscopy are :

(a) Instabilitry of sample during image acquisition. (b) Decaying of signal. (c) Live cells move and suffer photo-toxic damage. (d) Fluorescent probes may undergo saturation, photoconversion and detachment from target. (e) Fluctuation of intensity of excitation. (f) Drifting of focal plane. (g) Variation of environmental factors. (h) Chromatic aberrations etc.[32].

(h) Colony survival assay :

All the cell lines were cultured in 25 cm^2 flasks in a combination of 50 : 50 DMEM/F-12 medium containing 0.1 mM non-essential amino-acids, 20 mM HEPES and 10 mM bicarbonate containing 7.5% fetal calf serum and 7.5% newborn calf serum. For the fibrolasts experiments were performed below passage 18. Cells were grown to plateau phase to minimize changes in the cell cycle distributions during the experiments. Cells in plateau phase were fed 2–4 days before experimental procedures were started. Flow cytometry confirmed that cells were in plateau phase and showed no measurable change in cell cycle distribution during the experimental procedures.

For irradiation, X-ray unit operating at 250 kV_p with 1.07 mm Al filter was used giving a dose rate of 142 cGy/min. Cells were irradiated on ice to reduce repair during irradiation.

After final irradiation, cells were trypsinized, counted and plated for the colony survival assay. Cells were plated into 60 mm tissue culture dishes at numbers estimated to give 50 variable colonies. After 11–23 days, depending upon the cell lines, the dishes were rinsed, fixed, stained and the colonies counted. The survival values presented were adjusted to account for the survival of the inducing doses. All experiments were repeated three times. Flow cytometric technique was used to confirm that cells were in plateau phase and showed no measurable change in cell cycle distribution during the experimental procedures. Relative survival for various adapting doses of 0, 20, 50, 100, 150 and 200 cGy for a challenging dose of 2 Gy was calculated as[33] :.

$$\text{Relative survival} = \frac{\text{Survival after 2 Gy of adapted cells}}{\text{Survival after 2 Gy of non-adapted cells}}$$

(i) Electron spin resonance :

Electron spin resonance detects free radicals from the magnetic field induced by the non-paired electron characteristic of these species. It permits to identify the chemical nature of the radical and measure the kinetics of formation and decomposition of radicals. Fundamental basis and practical use of this technique has been reported[34]. Spin trapping agents can not be injected in humans for their toxicity effect. Some specific examples of spin trapping techniques are mentioned below :

By rapid collection of blood in a tube containing a spin trap agent, abnormal ESR signals are detected after repurfusion during coronary angioplastia, carbon centered free radicals are detected in coronary sinus blood from patients undergoing elective cardioplagia[35,36]. Ascorbate radicals may also be a marker of oxidative stress. Decrease in ascorbyl radical is found after aortic valve replacement[34-37].

(j) Chemiluminescence :

It is an effective detection method for oxidative stress *in vivo*. In this method a direct ultra weak emission of light by singlet oxygen can be measured by using a germanium photo-detector cooled by liquid nitrogen. The emission can be measured directly in cells or animals using a special apparatus with a red sensitive photo-multiplier. A few examples are : stress on hepatic microsomes, isolated hepatocytes and tissues. Whole heart after post ischemic oxygenation has been detected by chemiluminescence.

Luminol has been used to increase the sensitivity of detection (λ_{max} 480 nm) for various oxygen derivatives. Lucigenin is more specific for superoxide anions. Mostly membrane damages and mitochondrial damages are possible to be detected by using these probes. Chemiluminescence has also been used to detect phospholipids hydroperoxides mainly after HPLC separation. A special chemi-luminometer for gas permits the measurement of NO after extraction by a stream of inert gas and a reaction with ozone. Production of total blood after stimulation by PMA can be measured using luminol. This technique has been used in coronary angioplasty[38-41].

(k) Measurement of oxidative DNA base damage :

Measurement of oxidative base damage in tissues and cellular DNA remains a challenging analytical problem. For DNA having a concentration lower than 30 μg, the threshold of detection is close to one single lesion per 10^5 -10^6 normal bases. So, a high sensitivity is required. Given below a few oxidative DNA damage measuring methodologies along with their sensitivities and amount of DNA required for measurement[42] (Table 5).

Table 5

Method	DNA	Sensitivity	Amount of DNA (μg)
HPLC/ Electrochemistry	Hydrolysed	1×10^{-5}	25–50
HPLC/MS (Thermospray)	Hydrolysed	10^{-4} to 10^{-5}	30–40
Fluorescence	Hydrolysed	5×10^{-5}	4–8
GC/SIM-MS	Hydrolysed	1×10^{-5}	50–100
HPLC-P^{32} Post labelling	Hydrolysed	1×10^{-5}	1–5

(l) Salicylate hydroxylation :

The products of salicylate hydroxylation as an assay marker of oxidative stress in man has been considered here. This methodology can be used by those attempting to detect and measure hydroxyl radical generation in oxidative diseases such as diabetes, myocardial infarction, rheumatoid arthritis, cancer and ageing.

Methodology :

Chemicals namely aspirin, sodium salicylate, 2,3-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid and 3,4-dihydroxybenzoic acid were used at a stock solution concentration of 100 mg/L. The solvent used contained 85% phosphate buffer, 100 mM : 0.10 mM SDS, pH 3.3 and 15% methanol.

The chromatograph consists of two solvent delivery pumps, a sample injector, an analytical stainless steel column packed with ultrapure ODS 5 um (150 × 4.6 mm I.D.) a guard column packed with 10 μm spherical (30 × 4.6 mm I.D.) and a multi-wavelength detection system. The system was controlled by a computer. Salicylic acid assay was performed. Aliquots of 100 μL of standard solutions of plasma samples were mixed with 100 μL 2.5 μM 2,6-DHBA and deproteinized by 200 μL of ethanol in polypropylene conical tubes. The samples were mixed on a vortex type mixer for exactly 2 min. The tubes were then centrifuged at 1600 g for 15 min and 50 μL of the supernatant was diluted with 950 μL of mobile phase. The diluted solution was then filtered and 50 μL was injected onto the column. Mobile phase consisted of sodium citrate and acetate 30 mM, pH 5.45/methanol (85/15). Flow rate was 1 ml/min and the detector was set up at 295 nm. Standard curves were obtained from measurement of peak area ratios. Saline and plasma blanks were analyzed with each set of standards. The used concentrations of salicylic acid were 0 to 1000 μmoles/L of salicylic acid.

Aliquots of standard solution and of plasma samples (400 μL) were mixed with 100 μL of 2.5 μM 3,4-DHBA and acidified by 75 μL of concentrated hydrochloric acid in 10 × 70 mm glass tubes. The samples were mixed on a vorex type mixer for 30 s. 3 ml of ethyl acetate were then added and mixed for exactly 2 min. The tubes finally centrifuged at 1600x g for 15 min and 2 ml of ethyl acetate phase were dried under nitrogen stream. The dry residue was taken up by 200 μL of mobile phase and 100 μL were injected into the column. Mobile phase consisted of sodium citrate and acetate 30 mM, pH 3.90. Flow rate was 0.2 ml/min. Standard curves were drawn using peak area ratios. Saline and plasma blanks were analyzed with each set of standards. Lower concentrations were used for 2,3-DHBA (25-400 nMol/L) and higher concentration were used for 2,5-DHBA (125-2000 nMol/L).

Preparation of standard curves : Saline and plasma with no detectable salicylate as measured by the present method were used to make up solutions of different concentrations. Stock solutions of 2,3- and 2,5-DHBA respectively were stable for at least 2 months when stored at 4 °C in the dark. For salicylic acid assay, saline and plasma were used to make up solutions varying in concentrations (100 to 2000 μM from a stock solution of 100 mM). The concentrations were determined by the peak area ratio method using 3,4-DHBA as internal standard for DHBAS assay and 2,6-DHBA for salicylic acid assay.

Accuracy : The absolute recoveries of 2,3-DHBA added to a concentration of 25 nMol/L to six different plasma samples ranged from 86 to 102% with a mean of 94%. Similar studies on 2,5-DHBA (250 mole/L) yielded recoveries from 94 to 103% with a mean of 98%. Salicylic acid showed a recovery of 96.2%.

Human study : Salicylic acid, 2,3- and 2,5-DHBA concentrations were measured in 20 human subjects after a single oral dose (1000 mg in 150 ml water, prepared in just prior to administration) of soluble Aspegic. 7 ml blood samples were drawn from the ante-cubital vein with a vacutainer system in heparinized tubes before and 120 min following ASP (Aspirin) administration. The tubes were centrifuged within 30 min of blood collection at 1600 g for 10 min. Plasma was separated, quickly frozen and kept until required for analysis[43].

(m) Micronucleus assay :

A blood sample is taken before and directly after the exposure period of about 1 month. Chromosomal radiosensitivity is assessed *in vitro* by G_2-assay and G_0 micronucleus assay (MN). For the MN assay, a low dose rate (LDR) *in vitro* irradiation protocol was applied in addition to high dose rate irradiation of blood samples in order to determine the dose rate sparing effect (DRS effect). No statistically significant effect of the occupational exposures (up to 10 mSv) on the baseline MN frequencies without *in vitro* irradiation was observed. A comparison of the number of chromatid aberrations pre and post exposure shows no effect of the occupational exposure on the other hand the G_0-MN assay with low dose

rate irradiation protocol reveals a systematic reduction in chromosomal radiosensitivity by the exposure increasing with dose. For workers who received the highest dose (4–10 mSv) a statistically significant ($p < 0.05$) decrease of the *in vitro* induced MN yields and increase of dose rate sparing was observed.

Calculation :

The dose rate sparing (DRS) effect of LDR irradiation compared to HDR irradiation was calculated by the equation :

$$DRS = (1 - \gamma_{LDR}/\gamma_{HDR}) \times 100,$$

where γ_{LDR} is the MN yield at LDR and γ_{HDR} is the MN yield at HDR.

Micronuclei determination :

The incidence of micronucleus induction after radiation was assessed by cytokinesis blocking technique. Cytochalasin B was added to cells in culture immediately after the challenge dose of radiation to result in a final concentration of 3 μg/ml of culture medium. After 24 h incubation, cells were transferred to a microscopic slide, centrifuged at 100 g for 5 min, slides were air-dried and then fixed by flooding the slides with methanol : glacial acetic acid (3 : 1 v/v). Cells were stained with acridine orange (10 μg/ml in PBS) and micronuclei were scored using fluorescence microscopy[44,45].

(n) DNA double strand break assay :

For DNA double strand break assay, the CHEF (Clamped homogenate electric field) assay is very much suitable. In this technique the cells are grown to confluence and concurrently leveled with the addition of 0.02 μCi/ml[14] C-thymidine to the growth medium. After the experiment, the cells were suspended at a concentration of 10^6 cells per ml in a 1% agarose solution. These were placed in lysis buffer supplemented with 50 μg/ml proteinase K. The samples were placed in a dry bath incubator at 50 °C for 24 h. Following lysis the agarose plugs were washed with TE buffer (pH 8.0) for 1 h at 4 °C. This washing was repeated 3 times and the plugs stored in TE buffer at 4 °C until the CHEF electrophoresis was run. To run the CHEF electrophoresis, a CHEF DR II system was used complete with mini chiller. The gel was made with 0.08% agarose. Pulse field certified agarose dissolved in 1 × TAE buffer (pH 8.3). The plugs were placed in the wells and covered with 0.8% low melting point agarose to obtain a homogeneous environment for the DNA to migrate. DNA standards were also placed in random wells in order to provide controls for the electrophoresis. When the electrophoresis was complete the gel was stained using 10 mg/ml ethidium bromide. The gel was then cut for liquid scintillation counting, dissolved in scintillation cocktail, counted and analysed and the fraction of activity released was obtained[46].

(o) Comet assay :

About 10^4 cells either in a 5 μL blood sample or a 10 μL blood sample were mixed with 75–80 μL of warm low melting point agarose at 0.75%, 37 °C in a microfuge tube and spread over a fully frosted microscopic slide precoated with 200 μL of 0.1% agarose by layering a coverslip. The coverslips were gently removed after placing the slides on ice for 5 min. Slides were immersed in a jar containing a freshly prepared cold lysis solution (2.5 *M* NaCl, 100 mM EDTA, 10 mM tris, pH 10) to which were added 1% Triton X-100 and 10% DMSO just before use. The lysis was done at 4 °C for one hour at dark.

After lysis the slides were subjected to DNA unwinding for 20 min in a buffer (0.3 *M* NaOH, 1 mM Na_2 EDTA, pH > 13) and subsequently electrophoresed at 300 mA (15 V) for 20 min at 24 °C, under dim yellow light. Slides were washed twice for 5 min in a neutralizing buffer (0.4 *M* tris, pH 7.5) and stained with 75 μL propidium iodide (20 μL/ml). These were stored in a chamber at 5 °C and analyzed within 3 h under a fluorescent microscope with extinction at 530–560 nm, detection > 580 nm, coupled with an intensified target camera with a self designed image analysis system. The comet's images were analyzed using a software in BAS 1500. The intensity of the animal DNA damage was measured as tail moment, which was calculated by multiplying the tail length by the amount of DNA in the tail. The comet tail was set to be the area from the edge of the head to the end of the tail. Relative units were used for graphic presentation. For each sample about 80–100 images were analyzed[47].

(p) Fluorescence life time analysis :

This technique can detect DNA structural/topological damage in irradiated human lymphoid cells and it may

have potential application to *in vivo* bio-dosimetry and bio-monitoring. The lower detection limit is 0.1 Gy. Here the changes being monitored are persistent and are similar in the DNA changes for whole blood or lymphocytes. By detecting permanent damage, the technique may provide an estimation of radiation absorbed dose in exposed individuals. An early warning system capable of detecting pre-malignant changes in human genome would serve not only as a bio-dosimeter but also as a prognostic tool in screening for individual susceptibility and in the prevention, diagnosis and treatment of cancer.

Fresh whole blood collected from normal volunteer was mixed with phosphate buffered saline (PBS) at pH 7.4 and incubated for 0, 2 and 24 h at 37 °C. Following incubation lymphocytes were separated on a Ficoll-Hypacque density gradient, washed twice in ice cold PBS and re-suspended in ice-cold tris buffer at pH 7.4. The isolated lymphocytes or whole blood from each donor were divided into aliquots and irradiated (0, 0.1 and 1 Gy) at room temperature using an AECL Gamma Cell 220, Co-60 gamma ray source delivering a dose rate of ~1.0 Gy/min. After experimental treatments, lymphocytes were mixed with a glycine buffered solution adjusted to pH 10 with NaOH containing 1% Triton X-100, 0.05% RNAase and ethidium bromide (5 μg/ml). Samples were inverted three times, incubated on ice for 10 min and at room temperature for 5 min and inverted 3 more times. The resulting samples of intact nuclei ($\sim 10^6$ ml^{-1}) containing DNA intercalated EB were transferred to a 1 cm quartz cuvette at 4 °C for DNA conformational analysis using a PRA Inc., single photon counting fluorescence lifetime instrument.

A free running low pressure hydrogen (~50 kPa) flash lamp (50 kHz) operating at 6.0 Kv is used to interrogate the samples with excitation photons having a wavelength of 485 nm and the fluorescence emission at 610 nm is collected in a multichannel analyzer over 512 channels accumulating at least 5000 counts in the peak channel.

The fluorescence decay of the DNA-EB excited state complex is represented as a sum of exponentials, each with a defined pre-exponential term a_i and a lifetime π_i, where i is an integer, $I(t) = \Sigma a_i e^{-(t/\pi_i)}$.

Each individual experiment represents the fluorescence decay from excited EB molecules in a particular microenvironment. While the fluorescence yield of EB associated with each exponential component varies with the cellular mileau, according to its relative extinction coefficient, the extent of each molecular domain is quantitatively determined by the amount of each exponential component C_i which represents the area under the i-th component of the fluorescence decay curve.

$$C_i\ (\%) = (a_i\tau_i/T) \times 100$$

where $T = \Sigma_i a_i \pi_i$.

Following background subtraction the decay curves were de-convoluted from the lamp profile using the least squares method and analyzed in terms of a multi-exponential fit[48].

(q) Fluorimetric assay for measurement of oxidative stress in irradiated cells :

This assay is highly sensitive and capable of detecting increased dichloro-fluorescein (DCFH). Oxidation in the cells exposed to gamma irradiation at doses as low as 1.5 cGy with linear dose-response curves. But the dose-response curves varied considerably from one experiment to another and is influenced by the fluorescent substrate concentration and cell density. Since changes in DCFH oxidation may be related to changes that are related to oxidative stress in cells this assay can be useful to quantify radiation-induced oxidative stress and evaluate the efficacy of antioxidant agents in protection against radiation-induced oxidation.

Assay procedure :

Reagents, cells and cell culture media etc. : Dulbecco's modified Eagles medium (DMEM F12); phosphate buffered saline (PBS); 0.1% Trypsin; 0.1 mM EDTA tetrasodium solution; Epidermal growth factor (EGF); DCFH substrate etc. PBS was supplemented with 1 mM $CaCl_2$ and $MgCl_2$. A 100 mM DCFH stock was prepared in DMSO, stored frozen at -20 °C and diluted in PBS before use.

MCF-10 human breast epithelial cells were established. These were cultured in MEM/F12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 0.1 μg/m. Cholera toxin, 0 μg/ml insulin, 0.02 μg/ml EGF. The cells were sub-cultured by the treatment with trypsin-EDTA when the cells became confluent.

MCF-10 cells were cultured in 96 well strip plates

until the cells reached confluence. The cells were then washed twice with PBS and incubated for 30 min with 50 μM or 100 μM DCFH diluted in PBS to upload the cells with DCFH substrate. At the end of the incubation period, the cells were washed twice with PBS and then irradiated while the cells were covered with PBS at a volume of 50 μL per well. The sham irradiated strip well controls were removed from the plates prior to radiation exposure and placed back onto the plates after radiation exposure. ^{137}Cs source was used for gamma irradiation at a dose rate of 1.43 Gy per min for irradiating strip wells. The beam uniformity in the marked sample area in radiation chamber was checked by film exposure. Immediately after radiation exposure the plates were read with a Biotek Fl x 800 fluorescence plate reader at excitation and emission wavelengths of 485 and 525 nm respectively, three times at 90 s intervals using photo-detectors located underneath the plates. The reader sensitivity was set at 60. The level of cellular fluorescence measured in relative fluorescence units (RFU) and the rate of DCFH oxidation (ΔRFU/min) were both recorded. To determine whether the level of fluorescence measured in the assay system indeed reflects the change in the DCFH oxidation in cells and not the difference in DCFH-DA substrate uptake, ester cleavage or efflux of DCF/and or DCFH between the irradiated cells and sham irradiated control cells an additional experiment was performed in which DCFH-DA substrate solution (100 μM) was auto-oxidized in the dark at room temperature for 2 weeks, then diluted to a final concentration of 50 μM and incubated with confluent MCF-10 cells for 30 min on a 96 well strip plate. At the end of incubation period the cells were washed with PBS and irradiated while the covered with 200 μL PBS per well. At 2, 10 and 45 min after irradiation exposure the plate was read with fluorescence plate reader. The cells were washed after each plate reading and incubated with PBS at room temperature prior to next plate reading to allow the fluorescent substrate to diffuse across cell membranes to reach equilibrium. The levels of fluorescence measured for different dose groups were compared with that measured in the sham irradiated control group at each time after the radiation exposure to determine whether the radiation exposure affected the substrate uptake, ester cleavage or efflux of DCF and/or DCFH[49].

(r) Essence of γ-H2AX foci technique and DSB formation :

This assay is based on the finding that one of the highly conserved histone protein i.e. H2AX becomes phosphorylated at the sites of nascent DNA DSBs. The response is highly amplified and rapid involving the phosphorylation of hundreds to thousands of H2AX molecules within minutes on several mega-base equivalents of chromatin flanking the DSB. When visualized with an antibody the phosphorylated H2AX species, γ-H2AX, appears as nuclear foci[50].

Cell culture : Primary human fibrolasts from Lung, MRC-%, skin, HSF1, HSF2, 180BR etc. were grown in MEM supplemented with FCS and antibiotics.

Irradiation : Non-dividing confluent cell cultures were irradiated with 90 kV X-rays for doses up to 200 mGy, 1 mm Cu and 1 mm Al filters were used, thereby attaining a dose rate of 6–60 mGy/min. For higher doses the dose rate was 2 Gy/min! Dose rates were determined by ionization chambers and chemical dosimeters.

Immunofluorescence : For immune-fluorescence cells were fixed in 2% paraformaldehyde for 15 min, washed in PBS for 3 × 10 min, permeabilized for 5 min on ice in 0.2% Triton X-100 and blocked in PBS with 1% BSA for 3 × 10 min at room temperature. The cover slips were incubated with anti-γ-H2AX antibody for 1 h, washed in PBS, 1% BSA for 30 min and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular probes) for 1 h at room temperature. Cells were washed with PBS for 4 × 10 min and mounted by using vecta-shield mounting medium with 4,6-diamidino-2-phenylindole. Fluorescence images were captured by using a Zeiss Axioskop 2 Mot epi-fluorescent microscope equipped with charge coupled device camera and ISIS software. Optical sections through the nuclei were captured at 0.2 μm intervals and the images were obtained by projection of the individual sections. γ-H2AX foci were counted 3 min after irradiation. The plot of DSBs per cell, against dose shows a linearity from 0.001 Gy to 5 Gy (detected by immune-fluorescent study) and 5 Gy to 100 Gy by PFGE.

γ-H2AX foci are discretely different from apoptotic features (bubble like appearance of nucleus) or micronuclei.

Conclusion : This large number of techniques when applied meticulously, open the molecular, bio-macromolecular, cellular and genetic intricacies of the living system in un-irradiated and gamma-irradiated states for health and disease purposes of the society at large.

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