

RESEARCH ARTICLE

Peripheral Blood Lymphocytes as *In Vitro* Model to Evaluate Genomic Instability Caused by Low Dose Radiation

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Abstract

Diagnostic and therapeutic radiation fields are planned so as to reduce side-effects while maximising the dose to site but effects on healthy tissues are inevitable. Radiation causes strand breaks in DNA of exposed cells which can lead to chromosomal aberrations and cause malfunction and cell death. Several researchers have highlighted the damaging effects of high dose radiation but still there is a lacuna in identifying damage due to low dose radiation used for diagnostic purposes. Blood is an easy resource to study genotoxicity and to estimate the effects of radiation. The micronucleus assay and chromosomal aberration can indicate genetic damage and our present aim was to establish these with lymphocytes in an *in vitro* model to predict the immediate effects low dose radiation. Blood was collected from healthy individuals and divided into 6 groups with increasing radiation dose i.e., 0Gy, 0.10Gy, 0.25Gy, 0.50Gy, 1Gy and 2Gy. The samples were irradiated in duplicates using a LINAC in the radiation oncology department. Standard protocols were applied for chromosomal aberration and micronucleus assays. Metaphases were stained in Giemsa and 200 were scored per sample for the detection of dicentric or acentric forms. For micronuclei detection, 200 metaphases. Giemsa stained binucleate cells per sample were analysed for any abnormality. The micronuclei (MN) frequency was increased in cells exposed to the entire range of doses (0.1- 2Gy) delivered. Controls showed minimal MN formation (2.0%±0.05) with triple MN (5.6%±2.0) frequency at the lowest dose. MN formation increased exponentially with the radiation dose thereafter with a maximum at 2Gy. Significantly elevated numbers of dicentric chromosomes were also observed, even at doses of 0.1- 0.5Gy, compared to controls, and acentric chromosomes were apparent at 2Gy. In conclusion we can state that lymphocytes can be effectively used to study direct effect of low dose radiation.

Keywords: DNA damage - low dose radiation - CBMN assay - chromosomal aberrations - *in vitro*

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Introduction

Radiations are the part of day to day life and there is no low threshold limit established for the lowest safe dose which can pose no affect on the human genome (Cardoso et al., 2001). Nonetheless it is established that radiations however small in dose, cause double and single strand breaks in human genome and the recovery of DNA depends on the nature and quantity of DNA damage caused (Nikjoo et al., 2003). The genetic damage occurring due to radiation absorbed by any human is important to predict future exposures and to retrospectively asses the cause of genotoxicity. The risk evaluation and dose estimation is possible in cases of high radiation exposure and have been reported (Cardoso et al., 2001; Nikjoo et al., 2003; He et al., 2013). However the estimation methods for low dose radiation exposure and risk evaluation have not been fully developed. Risk estimates for low-LET (George et al., 2003) radiation protection purposes are

based mainly on epidemiological studies of populations exposed to substantial doses. Exposure to medical x ray, CT Scans (Brenner et al., 2001; Gonzalez et al., 2007; Pierce et al., 2012), Angiography (Johnson et al., 2014) have contradicting reports. It is difficult to estimate the coupled excess cancer risks by studying populations with exposures limited to the low-dose range. This is because, at low doses, the radiation-related excess risk, which is thought to be relative to dose is perhaps less when compared to risks at higher doses, and also there is variation in the background exposure level i.e. absence of exposure. Latest diagnostic procedures with low radiation involvement like diagnostic CT scan, Angiography, Mammography equipments have become routine and the patients undergoing the radiation based diagnostic procedures have increased considerably (Brenner et al., 2001; Gonzalez et al., 2007; Pierce et al., 2012).

Exposure to medical and diagnostic radiation exposure have contradicting reports debating on risks to benefit

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ratio. Keeping in view the appraisal that the risk to patients in the procedure is minimal and the benefit being the quick diagnosis and management of the disease, worldwide these tests are used for diagnostic purposes. The long lasting effects of the diagnostic radiations have not been evaluated and there are many controversial reports regarding the safety concerns of the patients undergoing the radiation based diagnostic test multiple times (Nguyen et al., 2011).

There are reports on dose estimations and methods to detect the genetic instability caused by the radiations and MN assay and presence of dicentric (Brooks et al., 1993), which considered as the gold standard technique to establish genotoxicity (M'kacher et al., 2015; Milalic et al., 2009). Micronucleus formation is a marker for early detection of DNA damage caused by ionising radiation. The study on radiation effects started after World War II and most works focused on animal models on a very large scale, tumor induction studies were then carried out in rats and mice over the next three decades. Cellular systems were developed in the 1970s to study malignant transformations of individual cells *in vitro*. Several researchers have highlighted the damaging effects of high dose radiation but still there is a lacunae in identifying the damage caused by low dose radiation used for diagnostic purposes, with the establishment of an *in-vitro* model we will be able to study the immediate effects low dose radiations have on human peripheral blood lymphocytes. The objective of the present study was to study *in-vitro* low dose radiation (<1Gy) effects on peripheral blood, by evaluating radiation effects on genomic (nuclear) and chromosomal integrity.

Materials and Methods

Sample collection and Dose estimation: About 15 ml of blood was collected from a healthy 35 yrs female in heparinised vial. The samples were irradiated using Linear accelerators (LINAC) at radiation oncology department of Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India. The sample was divided into 6 groups, in duplicates, with increasing radiation dose i.e., 0Gy, 0.10Gy, 0.25Gy, 0.50Gy, 1Gy and 2Gy.

Customization for Blood samples irradiation

ELEKTA infinity high energy Linear accelerator (LINAC) having 6, 10 and 15 MV of Photon energy and 4, 6, 10, 12, 15, 18 MeV of electron energy with kVCT facility was utilized for blood sample irradiation. The radiation dosimetric outputs were accredited by National secondary standards laboratory (SSDL), BARC Mumbai. The samples contained in the Culture tubes were provided a build-up of adequate water equivalent thickness for 6MV photon in the advance immobilization and mould room facility at the department of radiation oncology. 1.5 cm thick wax bolus with thermoplastic coverage was prepared for reproducibility of the sample and to provide a rigid support to prevent any deformity in transportation and positioning. The sample thus prepared and moulded in build water equivalent sheath was imaged using CT simulator (16 Slice, Siemens Make) and 3 mm slices were generated along the entire length of the sample

(Figure 1A, B, C, D).

Rigorous QA of CT scan both for qualitative and quantitative reproducibility were performed using international protocols such as AAPM or ICRU. CT number reproducibility in particular was considered important for any possible heterogeneity correction. The image of the sample using DICOM protocol were transferred to treatment planning system (Xio 3D TPS 5.0). The dose calculation with correction for heterogeneity of air, interface of wax bolus and vial was performed using superposition algorithm which has inbuilt mechanism for tracing of CT number and density correction. 3D dose evaluation was obtained and accuracy of dose was accessed using DVH protocol. The dose was prescribed at isocenter of the treatment field. The treatment plan data using the record and verification system (Mosaik 2.0, Elekta make) was transferred to the treatment unit. Before delivery of the dose machine specific and phantom specific QA protocols were performed. The reproducibility of the dose to the sample was verified using 2D array of detectors and Film dosimetry system. It was within the tolerance limit of +2%. To ensure the reproducibility of the dosimetric and positional parameters kVCT of sample phantom was obtained and compared with the DRR generated from the CT simulation procedure of the sample phantom. This ensured the dosimetric and positional accuracy of +0.1mm. Dose of 0.1, 0.25, 0.5, 1.0, 2.0 Gy was prescribed at isocenter of the treatment field to have a uniform dose distribution. The requisite monitor units were delivered and sample was transported from radiation oncology department of the institute for further analysis.

Cytokinesis-block micronucleus (CBMN): Irradiated blood sample (0.5ml) was cultured in Culture medium (PBMAX, GIBCO-12557) for 72hrs; by incubating at 37°C in 5% CO₂ incubator (ESCO) for 72 hrs. Cytochalasin-B (Himedia-RM7683) was added at 44hrs. Cultures were harvested at 72 hrs following a short hypotonic treatment (10min) in 0.56% KCl (GIBCO-10575-090) at room temperature. Cells were fixed 3-4 times in fresh, chilled Carnoy's fixative (methanol: acetic acid, 3:1) and left for overnight fixation. Cells were dropped over clean, pre-chilled slides, were air dried, and stained with Giemsa (GIBCO-10092-013); (5%, prepared in phosphate buffer, pH 6.8). The photographs of binucleated cells were taken and scoring was carried out at 100x magnification in Ziess, microscope. Micronucleus (MN) were identified and scored according to the criteria established by Fenech (Fenech, 2000). Proper aseptic conditions were maintained during the culture procedure.

Chromosome aberration assay (CA): Irradiated blood sample (0.5ml) was cultured in PB-MAX medium (GIBCO Cat. No. 12557-013) for 72hrs at 37°C in 5% CO₂ incubator (ESCO). KaryoMAX™ Colcemid Solution (GIBCO Cat. No. 15212) at concentration of 0.5µg/mL was added at 70hrs. Proper aseptic conditions were maintained during the culture procedure. Cells were harvested at 72hrs of culture, hypotonic shock in 0.56% KCl (37°C for 30min) was given to swell the cells. Cells were fixed 3-4 times in fresh, chilled Carnoy's fixative (methanol: acetic acid, 3:1) and left for overnight fixation. Cells were dropped over clean, chilled slides, air-dried stained

with Giemsa. The slides were analyzed on microscope (Metafer4 software-Zeiss) and Karyotypes were prepared (Moorhead et al, 1960). Metaphase pictures were taken at 100x resolution on Zeiss microscope and scoring was performed for presence of different aberrations, chromatid break (ctb), chromosome acentric fragment (csb), and chromatid exchange (cte).

Imaging

Microscopy was performed using CARL ZEISS Microscope. Images were acquired using axiocam image analyser using colour camera in transmitted light. Metafer 4 software was used for preparing Karyotypes. Images were stored at 100X objective lens. All slides analyzed in this study were coded and scored by two people independently.

Results

Dose delivery optimization

The radiation beam output of LINAC was calibrated both for conventional fields and small fields using 0.6cc and 0.015 cc PTW ion chamber. The latest protocol (TRS 398) of IAEA was utilized for beam library generation and calibration. Necessary correction for ambient temperature and air pressure were utilized as per protocol. The beam library for treatment planning system was thus generated and modelled based on actual measurements.

Effect of radiation dose on genomic stability- Micronuclei formation and other cytotoxic abnormalities

Among all the groups the percentage of dividing cells observed was almost similar and the number of dividing cells reduced significantly in 1Gy and 2 Gy groups as more number of apoptotic cells was observed (Table 1). Very low micronuclei formation was observed in control group (0.66 ± 0.06). The numbers of micronuclei significantly increased in 0.1Gy exposure group and thereafter an exponential increase in micronucleus was observed with the dose delivered. Some of the binucleated cells also depicted other genomic instability markers like formation of nuclear buds, nucleocytoplasmic bridges and polynucleate cells. These abnormalities were also scored along with the binucleated cells counted and are detailed in Table 1 (Figure 2). Ionizing radiation is able to produce a unique type of damage in which multiple lesions are encountered within close spatial proximity therefore in order to get clearer picture of DNA disruption we also focused on secondary structures of cytogenetic damage. There were no nuclear buds, nucleocytoplasmic bridges and polynucleate cells observed in lymphocytes of control group. The number of nuclear buds, nucleocytoplasmic bridges and polynucleate cells increased in lymphocytes of 0.1Gy, 0.25Gy and 0.5Gy exposure; however their number decreased in 1 and 2 Gy group, (not different from control). There were no apoptotic cells observed in unexposed, 0.1Gy and 0.25Gy irradiated cells, due to higher nuclear damage the number of apoptotic cells increased significantly in lymphocytes of 1Gy and 2Gy group. At relatively high doses (1 and 2 Gy) the damage is high and distinct but at low doses the types of aberrations

are complex their numbers are substantially lower but are still present.

Effect of radiation dose on chromosomal integrity: Induction of Chromosomal aberration

Chromosome aberration frequency is one of the most reliable biological markers to detect DNA damage by radiation exposure. To analyze chromosomal aberrations, more than 200 metaphases were scored per sample (400 metaphases per group) and assessed for dicentric, acentric chromosomes, fragments and any other related abnormality. The control value for dicentrics was $< 1/1000$.

Prominent radiation-induced changes in the frequency of chromosome aberrations was observed at the lowest dose (0.1Gy). The number and type of aberrations observed at 0.1Gy and 0.25 Gy were almost similar (Table 2, Figure 3). The dicentric fragments were the highest at 0.50Gy, which decreased exponentially as the radiation dose was increased. DNA fragmentation increased exponentially with increasing radiation dose, number of fragmented chromosomes was the highest at 2Gy depicting replicated damaged DNA. This group (2Gy) also marked the presence of acentric fragments, which again was not observed at lower doses (Table 2).

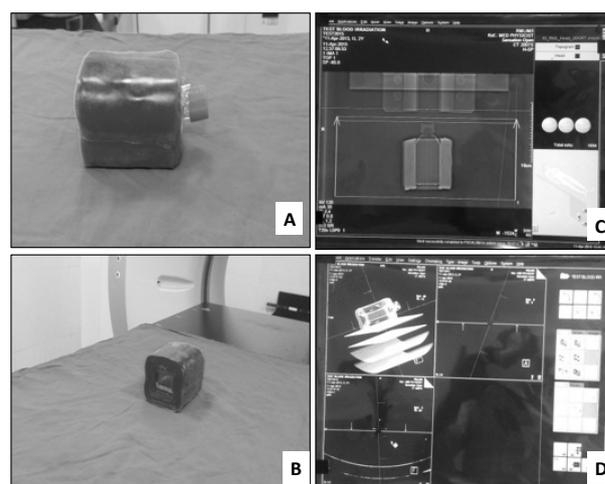


Figure 1. Irradiation of samples to different preset doses (A) and (B) Blood sample in culture tube with build up area of 1.5cm. (C) and (D) Irradiation of sample in LINAC

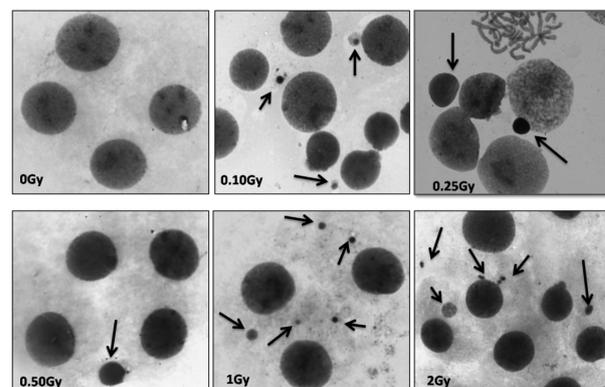


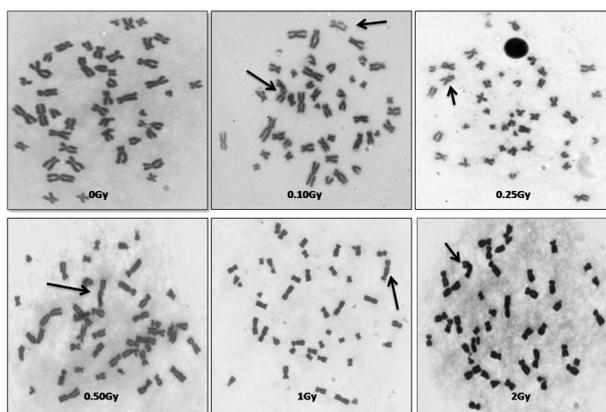
Figure 2. Micronuclei Observed at Different doses of Radiation

Table 1. Micronuclei Formation Induced in Human Lymphocytes by *in Vitro* Irradiation to Different Radiation Dose

Radiation Dose (Gy)	Bi-nucleate Cells Counted	%BN Cells	Micronuclei (MN)	Micronuclei (%)	Nuclear Buds	Nuclear Buds %	Nuclear Bridges	Nuclear Bridges %
0	300	40.3	2	0.66	-	-	-	-
0.1	550	38.2	28	5.09	25	4.55	30	5.45
0.25	500	38.89	31	6.2	28	5.6	26	5.2
0.5	633	38.88	62	9.79	15	2.37	35	5.53
1	543	36.4	93	17.13	9	1.66	36	6.63
2	428	35.08	122	28.5	-	0	-	-

Table 2. Chromosomal Aberrations Induced in Human Lymphocytes by *in vitro* Irradiation to Different Radiation Dose

Radiation Dose Gy	Metaphases Scored	Dicentric	Dicentric %	Fragments/Gaps	Fragments %	Acentric	Acentric %
0	200	0	0	0	0	0	0
0.1	222	15	6.75	3	1.35	0	0
0.25	202	15	7.42	4	1.98	0	0
0.5	252	18	7.14	0	0	0	0
1	234	10	4.27	6	2.56	0	0
2	201	6	2.98	33	16.41	6	0.99

**Figure 3. Chromosomal Aberrations (Dicentric) Induced in Human Lymphocytes by *in vitro* Irradiation to Different Radiation dose**

Discussion

In this study, peripheral blood lymphocytes were used to assess the genomic instability produced by exposure of low dose radiations on peripheral blood lymphocytes. In this study the genomic damage as low as 0.1Gy of radiation dose have been evaluated with the Micronucleus Assay and Chromosomal Aberration Assay. In the Micronucleus assay distinct micronucleus along with nuclear buds and nucleo-plasmic bridges were seen at doses as low as 0.10Gy. On increasing radiation dose secondary structure decreased and micronucleus formation increased. On the contrary in Chromosomal Aberration Assay DNA fragmentation increased with dose and the frequency of dicentric chromosomes were not different at the lower doses (<1Gy) and decreased thereafter on increasing dose to 1 and 2 Gy.

In our assay we have prominent damage at higher

doses (1 and 2 Gy) while secondary DNA damage structures were prominent at low doses (<1Gy). At higher dose levels there are fewer and damage tends to be large relative to baseline, and we are more likely to understand the causes of any substantial variation that might be confounded with radiation dose. It suggests that at low doses, the radiation-related risk, which is thought to be proportional to dose or somewhat less (non specific) and not distinct when compared to risks at higher doses.

Some studies interpret that radiation effects could arise, *de novo*, several generations after the damage inflicted. This observation of delayed response was termed radiation-induced genomic instability (RIGI).

Our understanding of risks associated with doses commonly encountered in daily life is not insignificant; we know, for example, that such risks are far lower than those observed in populations exposed to hundreds or thousands of mGy. The problem of quantifying risks that are so low as to be practically unobservable, and then recommending policies based on that quantification, is very difficult. When planning imaging experiments, especially when radiobiology and radiotherapy are involved, optimization of the CT protocol is strongly advised, to minimize the biologic consequences of the additional dose associated with the CT while maintaining sufficient resolution (Brenner et al., 2001; Gonzalez et al., 2007; Pierce et al., 2012). Paediatric CT too is a matter of concern as the exposed person has a larger period of time to develop mutation (Brenner et al., 2001; Miglioretti et al., 2012).

However the fundamental questions at the cell and molecular level to be considered for understanding risks at low doses are: 1) whether the damage caused by radiation is similar or distinct from endogenous damage; and 2) does damage occur at low doses/dose rates by ionizing radiation that cannot be repaired accurately; 3) is damage

induced under low dose and/or low dose rate conditions repaired by distinct mechanisms from damage induced at higher doses; and 4) are the signal transduction pathways activated by low dose and/or low dose rate conditions and what impact do these pathways have in determining the propagation or elimination of radiation damage in cells and tissues. One of the earliest stages in the cellular response to DSBs in the phosphorylation of the histone variant H2AX (He et al., 2013). By using a fluorescent antibody specific for the phosphorylated form of H2AX (γ H2AX) and immunofluorescence microscopy, one can visualize discrete nuclear foci at sites of DSBs. The formation and loss of γ H2AX foci after x-ray irradiation has been measured in human fibroblasts culture exposed to radiation doses as low as 1mGy, and focus yields have been shown to increase linearly with dose. This γ H2AX yield based method may be useful as a biologic dosimeter for diagnostic radiation exposure.

Early studies in biology related to radiation-induced cancer were largely descriptive in nature. This was mainly related to technical limitations in biological research. As such the ability to directly study low dose effects was limited. However, recent advances in techniques in cell and molecular biology are increasing the ability to directly approach these important questions. The current view is that the majority of radiation induced chromosome aberration are produced by the mismatch repair of DNA (DSB) quite possibly those involved in complex DNA lesions. It is also a well known fact that low doses of radiation can modulate the expression of a variety of genes thus studying and quantifying the damage is necessary considering the fact that genomic instability both chromosomal and mutational can be induced by high or low LET radiation (George et al., 2003; Brooks et al., 1993). The present study helps us establish an in vitro model to study radiation effects on humans and the type of changes it brings at the cellular and genomic level. However these results will be more universal if the groups can be further divided on the basis of age, sex, and occupation and eating habits.

Investigators in a study estimated that diagnostic x-rays use in the United Kingdom causes 0.6% of the cumulative risk of cancer to individuals until they reach age 75 years the equivalent of 700 cases per year, in which bladder cancer, colon cancer and leukemia are the most common malignancies. Recurrent CT can also pose serious threat to human health (Sodickson et al., 2009). To date, low-level radiation exposure from diagnostic x-rays has been monitored by using physical or chemical dosimeters. These sensitive devices can detect doses in the range of 0.1-50.0mGy. However, they determine only the exposure at a reference position outside the body and are unable to assess the actual dose deposited in the body. In vivo assessment of the biologic response to low level radiation exposure would represent an original approach to dosimetry. Ionising radiation induces a broad spectrum of damage in genes. Among these damage forms, DNA double-strand breaks (DSBs) are considered the most powerful lesions for cell killing, chromosome aberration formation, and cancer induction.

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References

- Brenner D, Elliston C, Hall E, et al (2001). Estimated Risks of radiation induced fatal cancer from paediatric CT. *AJR Am J Roentgenol*, **176**, 289-96.
- Brooks AL, Khan MA, Jostes RF, et al (1993). Metaphase chromosomal aberration as markers of radiation exposure and dose. *J Toxicol Environ Health*, **40**, 277-88.
- Cardoso RS, Takahashi-H S, Peitl P Jr, et al (2001). Evaluation of chromosomal aberrations, micronuclei, and sister chromatid exchanges in hospital workers chronically exposed to ionizing radiation. *Teratog Carcinog Mutagen*, **21**, 431-9.
- Fenech M. (2000) The in vitro micronucleus technique. *Mutat Res*, **455**, 81-95.
- George K, Willingham V, Wu H, et al (2002). Chromosome aberrations in human lymphocytes induced by 250 MeV protons: effects of dose, dose rate and shielding. *Adv Space Res*, **30**, 891-9.
- Gonzalez AB, Mahesh M, Kim KP, et al (2009). Projected Cancer Risks From Computed Tomographic Scans Performed in the United States in 2007. *Arch Intern Med*, **169**, 2071-7.
- He Y, Gong Y, Lin J, et al (2013). Ionizing radiation-induced γ -H2AX activity in whole blood culture and the risk of lung cancer. *Cancer Epidemiol Biomarkers Prev*, **22**, 443-51.
- Johnson JN, Hornik CP, Li JS, et al (2014). Cumulative radiation exposure and cancer risk estimation in children with heart disease. *Circulation*, **130**, 161-7.
- Miglioretti DL, Johnson E, Williams A, et al (2013). The use of computed tomography in pediatrics and the associated radiation exposure and estimated cancer risk. *JAMA Pediatr*, **167**, 700-7.
- Milacic S. (2009) Chromosomal Aberrations after exposure to low doses of ionising radiation. *J BUON*, **14**, 641-6.
- M'kacher R, E Maalouf, Terzoudi G, et al, (2015) Detection and automated scoring of dicentric chromosomes in nonstimulated lymphocyte prematurely condensed chromosomes after telomere and centromere staining. *Int J Radiat Oncol Biol Phys*, **91**, 640-9.
- Moorhead PS, Nowell PC, Mellman WJ, (1960). Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp Cell Res*, **20**, 613-6.
- Nguyen PK, Wu JC (2011). Radiation exposure from imaging tests: is there an increased cancer risk? *Expert Rev Cardiovasc Ther*, **9**, 177-83.
- Nikjoo H.. (2003). Radiation track and DNA damage. *Iran J Radiat Res*, **1**, 3-16.
- Pearce MS, Salotti JA, Little MP, et al (2012). Radiation exposure from CT scans in childhood and subsequent risk of leukaemia and brain tumours: a retrospective cohort study. *Lancet*, **380**, 499-505.
- Sodickson A, Baeyers P F, Andriole K P, et al.(2009). Recurrent CT, Cumulative Radiation Exposure and associated radiation induced cancer risks from CT of adults. *Radiol*, **251**, 175-84.