RESEARCH ARTICLE

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Quantitative Extra Long PCR to Detect DNA Lesions in Patients Exposed to Low Doses of Diagnostic Radiation

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Abstract

Background: Radiation causes oxidative lesions and strand breaks in DNA of exposed cells. Extended length PCR is a reliable method for assessing DNA damage. Longer DNA strands with DNA damage are difficult to amplify compared to smaller DNA strands by PCR. The present study was aimed to evaluate DNA damage caused by ionising radiation exposure in therapeutic and diagnostic medicine. **Materials and Methods:** The study group comprised 50 cases with low dose single exposure (LDS), low dose multiple exposure (LDM) and low dose angiography (LDA) which were compared with 25 high dose controls (HDC) and 25 controls with no exposure (NEC). Blood samples were collected within 1 hour of radiation exposure. DNA was isolated using a kit based protocol, 50 ng aliquots of DNA were used to amplify a long 13kbp DNA fragment of the β-actin gene by conventional PCR and band intensity was then quantified. Relative amplification was calculated and damage was expressed in terms of lesions per kilobase (kbp) by assuming a Poisson distribution. **Result:** Relative amplification was found to be 1.0, 0.87, 0.86, 0.72 and 0.69 with NEC, LDS, LDM, LDA and HDC groups, respectively. Cases undergoing angiography as well as high dose controls had high values, compared to NEC. The lesions/kbp calculated for LDS was 0.13, for LDM 0.15, for LDA 0.32 and for HDC 0.37 suggesting a linear increase in quantity with increasing radiation dose. **Conclusion:** DNA damage, even at low doses of radiation can be assessed by quantitative extra long PCR.

Keywords: DNA Damage- Q-PCR- Low Dose Radiation (LDR)- DNA Lesions

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Introduction

Low dose radiation were used in diagnostic radiology soon after the discovery of X ray in 1896 (Tubiana, 1996). The first Computed Tomography scan was invented in 1972, since then radiation based diagnosis has brought innumerable advances in the diagnosis and treatment process. Amidst all the benefits we get from diagnostic radiology we often forget about the after effects of exposure to ionising radiation (Bhattacharyya, 2016). The use of radiation in medical sources increased from 0.53 mSv in 1980 to 3 mSv in 2006 (NCRP report no 160), out of which CT scan alone accounts for 0.03mSv in 1980 to 1.47mSv in 2006 and is still increasing day by day (Wrixon, 2008). Since no radiation level higher than natural background can be regarded as absolutely "safe" the problem is to choose a practical level that, in the light of present knowledge involves negligible risk. As per International Commission of Radiological Protection guidelines the recommended safe dose of radiation exposure per year is 1mSv, excluding medical and occupational exposures (Schauer and Linton, 2009). The increasing use of these scans as a screening procedure in asymptomatic patients is an issue of concern for us as in low doses of radiation exposure, there is still considerable uncertainty about the overall effects. Ionising radiation are known to cause Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs) (Schipler and Iliakis, 2013) Although the DSB's and SSB's can be repaired, repair mechanism failure may occur in a small fraction of cases. Such cell may be eliminated by cell death however sometimes growth and cell division may occur resulting in replication of damaged /mutated DNA (Alberts et al., 2002). The DNA damage caused by ionising radiation mainly DSBs are hard to detect and differentiate if the damage is because of radiation exposure or some other carcinogen. Double strand breaks may lead to chromosomal aberrations, which affect cell viability and cause cell cycle deregulation (Van Gent et al., 2001), thus it becomes all the more important to study the damage caused by low dose radiation.

Several methods have been used to detect DNA damage by radiation viz., Single cell Gel Electrophoresis or comet assay, Halo, Terminal deoxyribonucleotidyltransferase – mediated deoxyuridine triphosphate Nick End Labeling (TUNEL), Chromosomal Aberration Assay (CA),

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Micronucleus Assay (MN) etc. (Christophe et al., 2010; Greve et al., 2012; Djuzenova et al., 2006; Nikitaki et al., 2015; Tewari et al., 2016). Quantitative Extra Long PCR has proved to be a sensitive technique to detect DNA damage and is the preferred method of choice by many researchers because of the nanogram quantity of DNA required. The main principle behind the quantitative PCR(Q-PCR) is that DNA lesions can slow down the progression of DNA polymerase on the target amplicon leading to decrease in the resulting PCR product as compared to the amplicon when the DNA is error free and intact. The longer the stretch of template DNA that is screened, the greater is the opportunity to detect differences in DNA integrity. This method has been explored to study the effects of several genotoxic agents (Jung et al., 2009; Santos et al., 2006; Hunter et al., 2010; Van Houten et al., 2000; Torres et al., 2000). As far as radiation is concerned the sensitivity of this method to detect DNA lesions has been explored in studies involving in vitro exposure to UV irradiation (Meyer et al., 2007). However this method is still unexplored when low doses of diagnostic radiation are concerned.

In the present study we have explored Q-PCR to detect DNA damage in patients undergoing scans involving radiation for diagnostic purposes. The present study was focussed to explore the application of PCR in quantitation of DNA damage caused by low dose ionising radiation in patients undergoing scans for diagnostic reasons. The low dose exposed samples were compared with negative controls (no exposure controls) and the positive controls (patients undergoing radiotherapy for therapeutic purposes).

Materials and Methods

Sample Collection

The study includes 100 cases with following groups Group I- No Exposure Controls (NEC), negative controls, Group II-Low Dose Diagnostic Radiation , which was further divided into three subgroups: Group II(A)-Low Dose Single Scan (LDS), Group II(B)-Low Dose-Multiple Scans (LDM) , and Group II(C)- Low Dose-Angiography (LDA), Group III-High Dose-Radiotherapy Patients (HDC), positive controls. The study was approved by the Institutional Ethical Committee (IEC No.7/14) at Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow. Informed Patient consent was taken from all the recruited participants and patient history was recorded, any past radiation exposure was also noted. 0.5 ml Intravenous Blood was collected in EDTA vacutainer and stored at -80 ^{o}C till further analysis.

Group I (N=50): Control population. Healthy people with no known radiation exposure were recruited to this group and prior consent was taken from all healthy subjects. It was also made sure that this population was also unexposed to other carcinogens, tobacco and alcohol and they do not have any family history of cancer.

Group II (A, B, C) (N=50): Low dose exposed population due to radiographic procedures for Medical use.

A: CT Scan (20)

B: Multiple Scans (15),

C: Angiography (single scanning) (15)

Group III (N=50): High dose exposed population. Patients who were undergoing radiotherapy treatment as part of cancer therapy were recruited as positive controls as the radiation is high in this group.

Exclusion criteria

Cases with history of taking alcohol, tobacco and other narcotics, cases with history of occupational radiation exposure, cases with family history of chromosomal genomic abnormalities, cases with previous exposure to radiological tests and treatment for malignancy, cases not giving consent to enter the study were excluded from the study group.

Radiation Quantification of Exposure

Group I: The patients with no known previous radiation exposure history were recruited in this group.

Group II: For Group II (A), In this group the patients were recruited from the department of radiodiagnosis of the Institute. Patients were undergoing Multi Slice Computed Tomography (MSCT) scan (64 Slice Philips make, Netherland) as per the prescription of the clinician. The Dose Length Product (DLP) CT radiation dose in mGy*cm received by the patient was recorded from the automated scanning system and blood sample was collected within 1 hour of MSCT scan. The DLP obtained was used to calculate the effective radiation dose. The guidelines of the International Commission of Radiological Protection (ICRP) publication 103 (Recommendations of International Commission of Radiological Protection published in 2007) was followed for the values of Tissue Weighing Factor for effective dose calculation. The formula used for the calculation of the effective dose is:

$$E = \sum_{z} \{ \sum_{T} W_T X H_T \}$$

where T is all ICRP-specified tissues and organs, W_T is the ICRP-specified tissue-weighting factor, HT is the dose to a particular organ or tissue, the inside summation T is over all tissues, and the outside summation Z is over all irradiated slabs (Christner et al., 2010).

Depending on patient size, body part scanned, number of sections taken and so forth , the effective dose may vary considerably, and so we recorded readings for individual patients. For Group II(B) where patients undergoing multiple scans were recruited the total radiation dose was calculated by adding the radiation dose of previous scans as well. Out of 15 patients, 14 cases in this group underwent multiple head scans (twice or thrice)and 1 underwent abdomen scan twice. For Group II (C) ,out of the 15 cases in this group, 9 were cardiac angiography, 4 were brain angiography, 1 neck angiography and 1 limb angiography.

Group III: For positive control, the records on linear accelerator (LINAC, Elekta, UK) in the department of radiation oncology of the institute was used for total dose calculation till the blood sampling day. The radiation dose varies in individuals according to the site of radiation therapy and dose constraints for various types and site

of tumors. Patients undergoing radiotherapy for various cancers like breast, lung, urinary bladder, head and neck tumors etc. were included in the study. The conversion of absorbed radiation dose to effective radiation dose was done as per the guidelines of ICRP Report no 103 published in 2007. The conversion factor for x-rays and gamma rays from Gray (Gy) to Seivert (Sv) are taken as 1 as per ICRP guidelines.

DNA Isolation and Quantitation

200 micro litre(µl) blood was used to isolate genomic DNA by QIAGEN Kit(Cat No.51304). The standard protocol recommended for the kit was used for the process. The Qiagen Kit has been recommended to amplify large targets as the DNA isolated is of high quality and easily reproducible (Furda et al., 2014). The DNA was stable for a long period of time and yielded comparable amplification results. The extracted DNA was then quantified using nanodrop spectrophotometer. The concentration of DNA in ng/ul was noted along with the optical density. The DNA was labelled and stored at -20 ^{o}C.

Extended Length PCR (XLPCR)

To maintain uniformity in PCR 50ng/ul DNA was used for amplification in all groups. The diluted DNA can be stored at -4 ^{o}C for several weeks

PCR Reagents

• Long PCR Enzyme Mix from Thermo Scientific (Cat No K0181); which includes Long Enzyme PCR mix (5U/ul) and 10x Long Buffer, 25mM $MgCl_2$ and water.

• 10mM dNTPs from Thermo Scientific (Cat No R0191) was purchased separately from Thermo Scientific having 2.5 mM of each nucleotide. Aliquots were made and stored at -20° C.

• Nuclear Long Primers for specific amplification of B actin Gene 13kbp fragment.(Primer Sequence listed in Table 1)Aliquots of the working primers (10µM) were made in proper aseptic condition and stored at -20° C.

PCR Reaction

To amplify a 13kbp B-actin gene we have established optimal concentration of reagents and as mentioned above, it might be noted here that different primers and different target genes require distinct concentration and condition. DNA amplification of B actin specific primers was done in a total volume of 50µl. The reaction mixture composition was 5µl of 10 X Long Enzyme buffer, 25mM $MgCl_2$, 100 picomole of each primer (nLong R and n Long F) 10mM Deoxy Nucleoside Triphosphate (dATP, dGTP, dTTP, and dCTP) and 2.5 units of Long PCR Enzyme mix. 1µl or 50ng of extracted DNA was added to every PCR mix, and the volume was made up to 50ul by nuclease free water. PCR mixtures were subjected to 40 cycles of amplification in an automated thermal cycler (Veriti, Applied Biosystems, USA). Thermal Profile and condition of PCR is mentioned in Table 2.

Agarose Gel Electrophoresis

An aliquot from PCR product of each sample was analysed by gel electrophoresis in 0.8 % agarose gel at constant voltage of 50V, along with 1 kbp DNA ladder. The Gel was visualized in the Gel Documentation system (BIORAD, USA) and bands corresponding to 13kbp of B actin DNA fragments were located in the gel (Figure 1). The Image was saved and exported for further analysis.

DNA lesions calculation

The agarose gel picture was used to quantify the band intensity of all samples for long PCR using Image J software. The mean intensity of each group was used for further analysis. For relative amplification the mean of normal samples was used to calculate the amplification in each radiation exposed group. These values were then used to calculate the lesion frequency per fragment at particular dose, such that lesions/strand at dose D=-ln AD/AC. This equation is based on the "zero class" of a Poisson expression. It is to be noted that poisson distribution requires an assumption that the lesions are randomly distributed.

Statistical Analysis

The results are presented as frequencies, percentages and mean±SD , the calculation of relative amplification was done using a Microsoft excel spreadsheet and the findings were based on these calculated values across all groups. Lesions/13kbp fragment were obtained and these values were used to calculate lesions/10kbp. As data was parametric and the comparison was between multiple groups, Tukey's Post hoc test was applied. Statistical difference ($p< 0.05$) between groups was determined.

Results

The mean (±SD) age and radiation dose was calculated for each group. The mean age and the male to female ratio of the test and control groups are depicted in Table 3. The age difference as well as the male to female ratio did not vary significantly across the groups. The mean (±SD) DLP radiation of test groups was calculated : Group II (A) received 1298.27±399 milliGray (mGy) of radiation, Group II (B) received 2484.97±1557, Group II (C) received the highest among low dose, 3813.92±2129 mGy, The controls groups comprising of high dose exposure in cases undergoing radiotherapy in Group III, received a radiation dose which was about 15 folds higher at 26011.20±19396 mGy. The mean effective radiation dose was found to be 23.58 mSv in Group II-A, 55.36 mSv

Table 1. Primer Sequence for Long PCR of β Actin Gene

Target Sequence		
nDNA long	Forward	5'-GCACTGGCTTAAGGAGTTGGACT-3'
nDNA long	Reverse	5'-CGAGTAAGAGACCATTGTGGCAG-3'

Table 2. Amplification Cycle for β-Actin Gene Amplification (13kbp).

S.No.	PCR Step	Temperature	Time	No. Of Cycles
1	Initial Denaturation	94°C	2 mins	
2	Denaturation	94°C	10 secs	} X 11cycles
3	Annealing	57°C	30 secs	
4	Extension	68°C	15 mins	
5	Denaturation	94°C	20secs	} X40 cycles
6	Annealing	57°C	30secs	
7	Extension	68°C	14(+5sec=n)mins	
8	Final Extension	68°C	1 min	
9	Hold	4°C	∞	

Table 3. Distribution of Age and Sex in Cases and Controls

Characteristics	Group I(NEC)	Group II(LD)			Group III(HDC)
		Group II (A)	Group II (B)	Group II (C)	
Age (yrs)					
Mean (±SD)	32.04 (±5.26)	43.26 (±3.21)	48.20 (±13.96)	46.54 (±16.02)	49.6 (15.64)
Range	26-48	17-75	24-70	17-70	35-80
Male:Female	11:14	14:06	10:05	11:04	19:06

in Group II-B and 272.71 mSv in Group II-C. The mean of calculated effective dose in Group III was 2336.04 mSv Table 4.

The relative amplification was found to be 1 in NEC, 0.87 in LDS, 0.86 in LDM, 0.72 in LDA and 0.69 in HDC. Cases undergoing angiography as well as high dose controls had significantly high values as compared to NEC. The lesions per 13 kilobase calculated in LDS were 0.13, in LDM 0.15, in LDA 0.32 and in HDC 0.37 suggesting a linear increase in quantity of DNA lesions with increasing radiation dose (Figure 2). The post hoc statistical analysis however showed no significant difference across groups but the difference in relative amplification can be counted as a major DNA damage

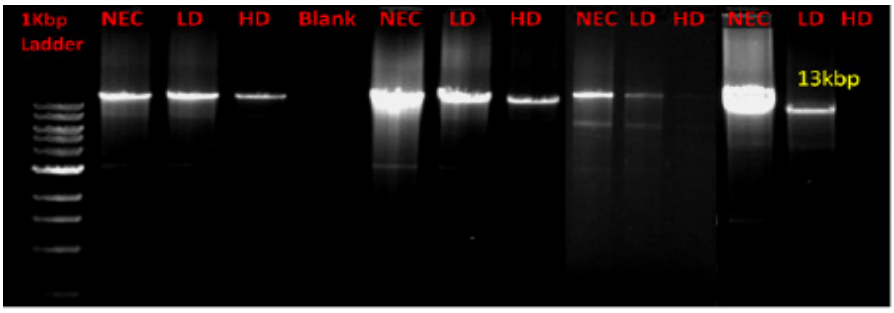

Figure 1. Long PCR Amplification of 13kbp Fragment of β-actin Gene. Agarose gel picture (0.8%) showing the PCR products from amplification of different DNA samples (NEC: No Exposure Controls, LD: Low Dose, HDC: High Dose Controls).

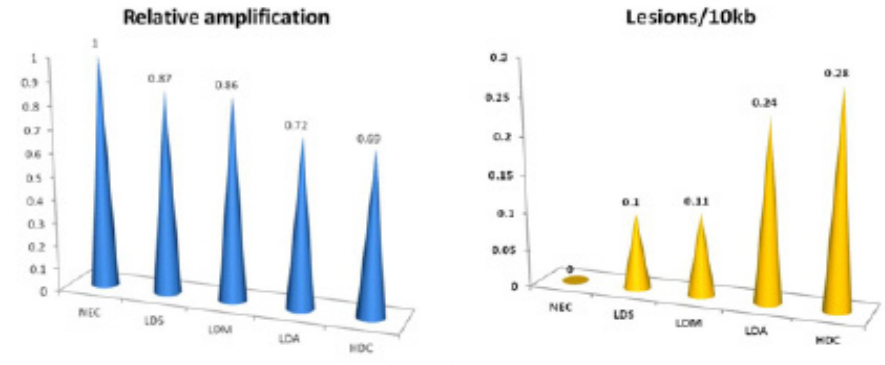

Figure 2. Diagrammatic Representation of Relative Amplification and Number of Lesions per 10kbp in Cases and Controls (NEC: 1 and 0, LDS: 0.87 and 0.1, LDM: 0.86 and 0.11, LDA: 0.72 and 0.24, HDC: 0.69 and 0.28 respectively)

Table 4. Showing Relative Amplification and Lesions/10kb in Cases and Controls

Sample	Radiation Dose(mSv)	Amplification Mean-Long	Relative Amplification	Lesions/13kbp
Group I(NEC)	0	106.66	1	-
Group II-A(LDS)	23.58	93.109	0.87	0.13
Group II-B(LDM)	55.36	92.24	0.86	0.15
Group II-C (LDA)	272.71	77.26	0.72	0.32
Group III (HDC)	2336.04	73.68	0.69	0.37

Table 5. Tukey's Post hoc Analysis between Cases and Controls.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I NEC	HDC	0.30920	0.14003	0.186	-0.0802	0.6986
	LDSE	0.12616	0.14304	0.903	-2716	0.5239
	LDM	0.13520	0.16169	0.919	-0.3144	0.5848
	LDA	0.27503	0.17387	0.513	-0.2085	0.7585
Group III HDC	NEC	-0.30920	0.14003	0.186	-0.6986	0.0802
	LDSE	-0.18304	0.14304	0.704	-0.5808	0.2147
	LDM	-0.17400	0.16169	0.818	-0.6236	0.2756
	LDA	-0.03417	0.17387	1.000	-0.5177	0.4493
Group II-A LDSE	NEC	-0.12616	0.14304	0.903	-0.5239	0.2716
	HDC	0.18304	0.14304	0.704	-0.2147	0.5808
	LDM	0.00904	0.16431	1.000	-0.4479	0.4660
	LDA	0.14888	0.17630	0.916	-0.3414	0.6391
Group II-B LDM	NEC	-0.13520	0.16169	0.919	-0.5848	0.3144
	HDC	0.17400	0.16169	0.818	-0.2756	0.6236
	LDSE	-0.00904	0.16431	1.000	-0.4660	0.4479
	LDA	0.13983	0.19174	0.949	-0.3934	0.6730
Group II-C LDA	NEC	-0.27503	0.17387	0.513	-0.7585	0.2085
	HDC	0.03417	0.17387	1.000	-0.4493	0.5177
	LDSE	-0.14888	0.17630	0.916	-0.6391	0.3414
	LDM	-0.13983	0.19174	0.949	-0.6730	0.3934

factor in low dose exposed population (Table 5).

Discussion

In the present study we have explored the Quantitative PCR based measurement of nuclear DNA damage for detection of DNA damage in cases undergoing diagnostic radiological examinations (CT, Angiography) single or multiple times. The low dose cases were compared with high dose patients undergoing radiotherapy treatment and no exposure controls, positive and negative controls respectively. The protocol followed was taken from the protocol of Furda et al published in 2014 (Furda et al., 2014) with some minor changes as per our study design. We observed that the DNA lesions were found to be increasing linearly with increasing radiation dose (Figure 2), the lesions were found to be maximum in HDC (Group III), and minimum in patients who underwent single CT scanning procedures.

The method of Q-PCR has been used for genotoxicity assessment in several other studies mainly focussing on eco-toxicology and chemical toxicology, age related studies, disease conditions and to study diabetic retinopathy (Jung et al., 2009; Trnka et al., 2009; Jung et al., 2009; Meyer, 2010; Wang et al., 2009; Wang et al.,2008; Meyer et al., 2007; Torres K et al.,2009; Santos et al., 2011; Tewari et al., 2012) but has not been used to study the damage post exposure to ionising radiation. Although several other methods have been used to quantify the immediate DNA damage (Christophe et al., 2010; Greve et al., 2012; Djuzenova et al., 2006; Nikitaki et al., 2015; Tewari et al., 2016) the requirement of only nanogram quantity of genomic DNA and the accuracy of a PCR reaction makes this method advantageous over others. We used 50ng of genomic DNA for amplification of a 13kbp fragment. This method is highly sensitive because of the use of the long PCR methodology that permits the quantitative amplification of fragments of genomic DNA between 10 and 25kb in length (Torres et al., 2000; Van Houten et al., 1998). This results in detection of low levels (1 per 105 kb) of lesions thus quantifying the DNA damage at specific gene loci. Any gene that can be amplified by PCR can be studied by this method. In the present study we have used a 13kbp fragment of Beta actin gene since it is a housekeeping gene and its expression will be the same

in all groups. Q-PCR method has been used to measure DNA damage at nuclear and mitochondrial level to study genotoxicity in a wide variety of cells and tissues (Furda et al., 2012; Hunt et al., 2016; Roy et al., 2011) Our study group where radiation induced DNA damage was studied the environmental exposure was taken to be same for all groups since the study population was urban and from the same region.

The natural background radiation of 2.4mSv was also common in all groups including cases (LDS, LDM, LDA) and controls (NEC, HDC). The relative amplification was calculated for each sample and the mean relative amplification was found to be decreasing with increasing exposure to radiation (Table 4). When the lesions per 10kbp was calculated using a poisson's distribution it was found to be 0.1 in LDS, 0.11 in LDM, 0.24 in LDA, which was the highest in low dose group (Group II) and 0.28 in high dose control group (Group III). This trend suggests that the quantity of lesion per 10kbp increased linearly with the increase in radiation dose. The results however were not found to be statistically significant. This method can be used to quantify the DNA lesions, if any, post exposure to ionising radiation however before concluding anything this has to be verified by studying the persistence of these changes.

This study was an attempt to explore the Q-PCR technique in efficient detection of DNA damage caused by radiation. Q-PCR is an established biomarker for the DNA damage detection and has been used to detect damage in various fields as mentioned earlier. The PCR assay has been used widely since 1990 to measure DNA damage and repair kinetics in nuclear and mitochondrial genomes after genotoxin exposure. This assay however was not explored to detect DNA damage caused by low dose radiation, few attempts have been made to study mitochondrial damage in 60 Cobalt radiation exposure (Wang et al., 2003). Although the traditional methods are more specific as well as sensitive, this new method can also be used as it has a few advantages over the traditional methods. The amount of blood required is minimal (200µl), the blood or even DNA can be stored for months as opposed to established cell culture techniques where the blood has to be processed immediately and is also time bound. The PCR also proves to be a low cost and not labour intensive as compared to the labour intensive and expensive techniques. Thus, scrutinizing the advantages of this technique the author will recommend this technique for an approximate DNA damage estimation but not an accurate DNA Damage estimation where radiation induced DNA damage is concerned.

In conclusion, DNA damage, even at low dose of radiation can be assessed by Quantitative extra long PCR.

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