Micronucleus frequency in peripheral blood lymphocytes of rats exposed to high background natural radiation

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ABSTRACT *Manavalakurichi, along the west coast of Tamil Nadu, India is well known for its high background natural radioactivity. The coastal belt is rich in monazite containing thorium, and the radiation dose in random spots has been recorded up to 142.6 mGy y^{-1}. One-month-old Wistar rats were exposed to natural background radiation, at the dose of 92.91±3.89 mGy y^{-1} for a period of 18 months and a parallel control set exposed to dose of 2.38 ± 0.26 mGy y^{-1}) was also maintained. Blood samples were collected from the rats (n=30) exposed to sand collected from a high background natural radiation area (HBNRA) and from rats (n=15) exposed to sand collected from normal background radiation area (NBRA) at intervals of 3 months up to 18 months, and were subjected to the cytokinesis-block micronucleus (CBMN) assay. The frequencies of micronuclei in samples of rats exposed to HBNRA and those exposed to NBRA sand were not significantly different ($P > 0.05$). However, a significant ($P < 0.05$) age-dependent increase in MN frequency was observed among both sets of rats. Moreover, no dose-dependent increase in MN frequency was seen in rats exposed to HBNRA.*

Keywords: *Background radiation, Micronuclei, HBNRA, Manavalakurichi*

Introduction

There are some areas in the world, where the natural background radiation level is high due to naturally occurring radionuclides in soils. Manavalakurichi, along the west coast of Tamil Nadu, India a high background natural radiation area (HBNRA). This region is rich in monazite containing thorium, and radiation dose in random spots have been recorded up to 142.6 mGy y^{-1}. The world average of effective dose from background radiation is about 2.4 mSv y^{-1} (ICRP, 1991; 2007; UNSCEAR, 2000a). Singh et al. (2007) studied the radioactivity in the southern Tamil Nadu and reported an average of 11.40 µGy h^{-1} (99.93 mGy y^{-1}) in coastal belt of Manavalakurichi. The HBNRAs provide an unique opportunity to study the health effects of chronic low level radiation exposure and is considered to be of high interest.

Cytogenetic analyses have been significant tools for studying the mutagenic effects of ionizing radiation, and micronucleus assay is one of the few methods available for screening the alterations to the entire genome of individual cells. The micronucleus assay in peripheral blood lymphocytes was first reported by Fenech and Morley (1985). The cytokinesis-blocked micronucleus (CBMN) assay is a comparatively less tedious and simple method for scoring chromosomal damage (Das and Karuppasamy, 2009). Analysis of the induction of micronuclei (MN) in lymphocyte cultures has indicated that the most convenient stage to score MN is during the interphase, where the cells have completed one cell division and are therefore capable of expressing MN (Fenech and Morley,1985).

A number of studies have been conducted for quantifying chromosome damage using MN as an alternative approach, because it provides a measure of both chromosome breakage and chromosome loss (Schmid, 1975; Countryman and Heddle, 1976). In cultured lymphocytes, it is one of the most consistent and specific method for assessing radiation- induced chromosome damage (Fenech and Morley, 1985; Fenech, 1993, 2000; Müller et al., 1996; Fenech et al., 2003; Gutiérrez-Enrí and Hall, 2003). In this study, the effect of chronic low-dose radiation was assessed by measuring the frequencies of MN in male Wistar rats exposed to HBNRA and NBRA sand.

Materials and Methods

Sampling location

Two sampling locations that showed high and low activity (measured from 1 m above ground level) reading on a radiation survey meter were chosen. The high-level radioactivity soil samples were taken from

Manavalakurichi (8°08′140″N, 77°18′154″E) and normal-level radioactivity soil samples from Rajakkamangalam Thurai (8°06′519″N, 77°22′374″E).

Collection of sand

Monazite-containing black sand sample and normal sand sample were scooped out from a depth of 5–30 cm from high and low activity sampling points using a clean, pointed hand shovel. About 100 kg of sand was collected from each location for preparation of sand beds. The samples were collected in clean polythene bags for easy transportation to the laboratory.

Preparation of sand bed

Two rectangular tanks of dimension 250 × 150 × 150 cm (L × B × H) were made of concrete and filled with sand up to a height of 60 cm (monazite-containing black sand and normal sand in two different cages). Above the sand a polythene sheet was spread to prevent animals from direct contact with the sand. The tanks were kept in an animal house with the temperature maintained at 25±3°C.

Measurement of radiation level

The radiation levels of sand beds were recorded at the height of 0.5 m using the micro-R Survey meter (Nucleonix) measuring micro-Sievert per hour ($\mu Sv\ h^{-1}$), with an accuracy of ±10, calibrated from Environmental Survey Laboratory, Kudankulam, Tamilnadu.

Animals

Male Wistar rats (outbred albino) aged between 2 and 3 weeks weighing ~100 g were studied. The rats were kept in the animal house in well ventilated acrylic cages with adequate water and rat feed until 1 month of age. The rats were tagged with coded metal ear tags made up of nickel-copper alloy for identification. The animals were then transferred to HBNR and NBR sand beds. Thirty rats were allotted to HBNRA sand bed and the rest (15) to NBRA sand bed The rats were exposed to these beds for 18 months.

Blood collection and processing

Peripheral blood was collected from the retro-orbital sinus of rats using standard heparinized micro-hematocrit capillary tubes. The blood sample withdrawn at one time from this location is 1% of the animal's body weight (e.g., 2.0 ml from a 200 g adult rat). as rats have to be anaesthetized for retro-orbital bleeding, ketamine (75 mg/kg) was injected intraperitoneally. Prior to and during the procedure the respiratory rate, response to noxious stimulus, spontaneous movement and anaesthesia recovery parameters were monitored at 5-min intervals. During recovery from anesthesia, respiratory rate, movement and ability to maintain sternal recumbency were monitored at 5-min intervals until the animal was ambulatory. The animals recovered within 30-60 min of the procedure. Samples taken in lithium heparin tubes were mixed by gentle inversion 5 to 10 times immediately after drawing and this was done to ensure homogenous mixing of the additives with the blood.

Blood collection interval

Blood samples were collected every 3 months up to 18 months. A total of six blood collections were made per animal. The quantity of blood withdrawn ranged between 0.5 and 1 mL. As the number of animals declined during subsequent collections due to increased mortality because of unknown reasons, the sample size is not equally distributed in the different time intervals.

Cytokinesis-block micronucleus (CBMN) assay

A volume of 0.5 mL of whole blood sample was added to 4.5 mL RPMI-1640 (Sigma-Aldrich) supplemented with L-glutamine, 30% heat-inactivated fetal bovine serum and antibiotics (penicillin/streptomycin – 100 U/ml) in a sterile culture flask with screw cap. 100 µL of phytohaemagglutinin (PHA), at the concentration of 20 µg/mL, was added to the culture tube. The flasks were kept in a slanting position at 37°C in an incubator. 20 µL of cytochalasin-B (Cyt-B) was added to the culture, 24 h post-PHA stimulation, to give a final concentration of 6 µg/mL. The culture was terminated 72 hours post-PHA stimulation. The cells were centrifuged at 1000 rpm for 10 min and the supernatant was removed. The cells were hypotonically treated with 7 mL of cold (4°C) 0.075 M potassium chloride (KCl) and centrifuged immediately at 1000 rpm for 10 minutes. Again the supernatant was removed and replaced with 5 mL freshly prepared fixative consisting of methanol:acetic acid (3:1). The fixative was added whilst agitating the cells to prevent the formation of clumps. The cells were washed with freshly prepared fixative two to three times and the supernatant 1 cm above the cell pellet removed; cells were resuspended gently, and the suspension dropped onto clean glass slides and allowed to air-dry. The slides were stained in 4% Giemsa's azur-eosin-methylene blue solution (Merck) in HEPES buffer (0.03 M; pH 6.5) for 15 min under dark conditions, followed by a quick rinse in distilled water and air dried. The dried slides were used for light microscopy analysis. 1000 binucleated cells with and without MN were counted per sample and recorded in the excel worksheet.

Statistical Analysis

All the calculations were done using the statistical software SPSS v11. ANOVA was used to compare the frequency of MN between HBNRA and NBRA-sand exposed groups. The frequency of MN at different age groups in HBNRA and NBRA exposed rats was also analyzed using ANOVA. Bonferroni *post hoc* test was applied for the multiple-comparison of age groups.

Results

The radiation dose at different time intervals to rats was calculated (Table 1). The distributions and frequencies of MN in peripheral blood lymphocytes of rats exposed to HBNRA and NBRA sand are shown in Table 2. In HBNRA sand-exposed rats, the frequency of MN was observed to be 15.0±2.9, 16.7±4.4, 17.2±4.6, 17.1±4.9, 18.5±5.1 and 20.3±4.4 per 1000 BN cells, for the dose rate of 23.23±0.97 mGy (3 months), 46.46±1.95 mGy (6 months), 69.69±2.92 mGy (9 months), 92.91±3.89 mGy (12 months), 116.15±4.86 mGy (15 months) and 139.38±5.84 mGy (18 months), respectively, whereas it was 15.3±3.0, 16.4±3.9, 16.9±3.7, 17.5±3.8, 17.9±3.7 and 19.7±3.8 per 1000 BN cells from NBRA sand-exposed rats for the dose rate of 0.60 ± 0.07 mGy (3 months), 1.19 ± 0.13 mGy (6 months), 1.79 ± 0.20 mGy (9 months), 2.38 ± 0.26 mGy (12 months), 2.98 ± 0.33 mGy (15 months) and 3.58 ± 0.40 mGy (18 months), respectively (Table 3, Fig. 1). No significant difference was seen in MN frequency between HBNRA and NBRA sand-exposed rats ($P > 0.05$) at any of the time intervals.

However, an age-dependent increase in the frequency of MN was observed (Table 4). Analysis of variance suggested that the difference in frequencies were statistically significant among age groups ($P = 0.001$). *Post-hoc* analysis of MN frequencies in the age groups revealed that the mean difference was significant at the 0.05 level. Linear regression analysis of the data also showed a significant increase in the frequency of MN with respect to age (Fig. 2). The age-related increase in the frequency of MN in rats was similar in HBNRA and NBRA-sand exposed rats($P = 0.041$).

Table 1

Measurement of radiation dose absorbed by rats in different time intervals

Sand bed	Radiation dose (mGy y^{-1})		Radiation dose absorbed by rats in different time intervals (months) mGy (Mean±SD)					
	Mean	SD	3	6	9	12	15	18
HBNRA	92.91	3.89	23.23± 0.97	46.46± 1.95	69.69± 2.92	92.91± 3.89	116.15± 4.86	139.38± 5.84
NBRA	2.38	0.26	0.60± 0.07	1.19± 0.13	1.79± 0.20	2.38± 0.26	2.98± 0.33	3.58± 0.40

Table 2

Distribution of number of micronuclei per 1000 bi-nucleated cells exposed to HBNRA and NBRA sand

	Exposure duration (months)	N	Radiation Dose (mGy) Mean±SD	BNC	MN	Distribution of MN			MN (%)
						1	2	3	
HBNRA-sand-exposed	3	30	23.23 ± 0.97	29567	451	416	16	1	1.5
	6	30	46.46 ± 1.95	29529	502	442	27	2	1.7
	9	29	69.69 ± 2.92	28542	400	418	38	2	1.8
	12	27	92.91 ± 3.89	26578	462	384	36	2	1.7
	15	26	116.15 ± 4.86	25558	482	405	34	2	1.9
	18	24	139.38 ± 5.84	23557	487	403	36	4	2.1
NBRA-sand-exposed	3	15	0.60 ± 0.07	14780	229	211	9	0	1.5
	6	15	1.19 ± 0.13	14766	246	223	10	1	1.7
	9	15	1.79 ± 0.20	14765	253	218	16	1	1.7
	12	14	2.38 ± 0.26	13769	245	218	12	1	1.8
	15	14	2.98 ± 0.33	13766	251	217	17	0	1.8
	18	12	3.58 ± 0.40	11780	236	204	14	1	2.0

N – number of rats; BNC – bi-nucleated cells; MN – micronuclei; HBNRA – High background natural radiation area; NBRA – Natural background radiation area

Table 3

Influence of background radiation on MN frequencies in rat peripheral lymphocytes at different timelines

Exposure duration (months)	HNBRA sand exposed				NBRA sand exposed				P value
	Radiation dose (mGy)	N	MN/1000 cells	MN/cell	Radiation dose (mGy)	N	MN/1000 cells	MN/cell	
3	23.23 ± 0.97	30	15.0±2.9	0.015±0.003	0.60 ± 0.07	15	15.3±3.0	0.015±0.003	0.802
6	46.46 ± 1.95	30	16.7±4.4	0.017±0.004	1.19 ± 0.13	15	16.4±3.9	0.016±0.004	0.805
9	69.69 ± 2.92	29	17.2±4.6	0.017±0.004	1.79 ± 0.20	15	16.9±3.7	0.017±0.004	0.785
12	92.91 ± 3.89	27	17.1±4.9	0.017±0.005	2.38 ± 0.26	14	17.5±3.8	0.018±0.004	0.797
15	116.15 ± 4.86	26	18.5±5.1	0.019±0.005	2.98 ± 0.33	14	17.9±3.7	0.018±0.004	0.695
18	139.38 ± 5.84	24	20.3±4.4	0.020±0.004	3.58 ± 0.40	12	19.7±3.8	0.020±0.004	0.680

HBNRA – High background natural radiation area; NBRA – Natural background radiation area; N – number of rats; MN – micronuclei

Table 4

Influence of age on MN frequencies in rat peripheral lymphocytes exposed to HBNR and NBR sand

Age (months)	HNBRA sand exposed					NBRA sand exposed				
	Radiation dose (mGy)	N	MN/1000 cells	MN/cell	P value	Radiation dose (mGy)	N	MN/1000 cells	MN/cell	P value
4	23.23 ± 0.97	30	15.0±2.9	0.015±0.003	0.001	0.60 ± 0.07	15	15.3±3.0	0.015±0.003	0.041
7	46.46 ± 1.95	30	16.7±4.4	0.017±0.004		1.19 ± 0.13	15	16.4±3.9	0.016±0.004	
10	69.69 ± 2.92	29	17.2±4.6	0.017±0.004		1.79 ± 0.20	15	16.9±3.7	0.017±0.004	
13	92.91 ± 3.89	27	17.1±4.9	0.017±0.005		2.38 ± 0.26	14	17.5±3.8	0.018±0.004	
16	116.15 ± 4.86	26	18.5±5.1	0.019±0.005		2.98 ± 0.33	14	17.9±3.7	0.018±0.004	
19	139.38 ± 5.84	24	20.3±4.4	0.020±0.004		3.58 ± 0.40	12	19.7±3.8	0.020±0.004	

HBNRA – High background natural radiation area; NBRA – Natural background radiation area; N – number of rats; MN – micronuclei

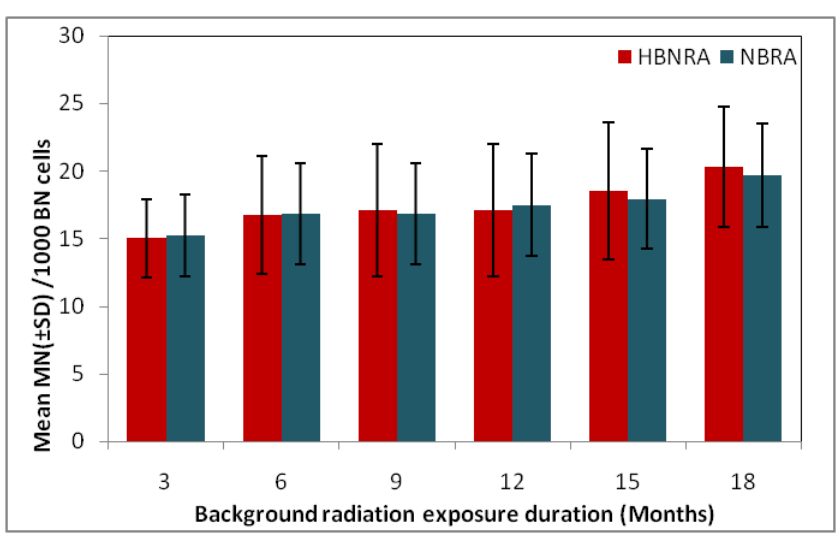

Fig. 1. Influence of background radiation on MN frequencies among HBNRA and NBRA sand exposed rats.

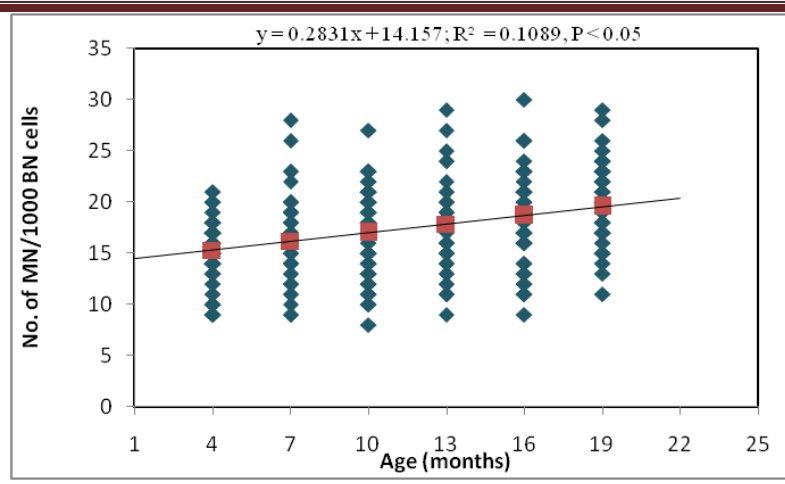

Fig. 2. Influence of age on MN frequencies among HBNRA and NBRA sand exposed rats.

Discussion

Micronuclei (MN) in mammalian cells serve as a reliable biomarker of genomic instability and genotoxic exposure. Increased MN frequency is commonly observed in cells bearing intrinsic genomic instability and in normal cells exposed to genotoxic agents, including ionizing radiation. Micronuclei arise from acentric chromosome fragments or from lagging whole chromosomes and CBMN assay provides a unfailing estimate to these types of chromosome damage. In the present study, the CBMN assay was used to estimate the MN frequencies in rats exposed to HBNRA and NBRA sand. No significant diffrence was seen between the frequency of MN in HBNRA and NBRA sand exposed rats.

Similar to the results of this study, Karuppasamy et al. (2016), Thampi et al. (2005) and Cheriyan et al. (1999) reported statistically comparable frequencies of MN and dicentrics among individuals in high background radiation areas of Kerala. Mohammadi et al. (2006) reported no significant difference in basal MN frequencies between individuals from high background radiation areas of Ramsar, Iran and control areas.

However age-dependent increase in MN was seen in this study. It has been reported that, in old age, biomarkers of genomic instability, such as MN, are more common in the peripheral lymphocytes (Joseph et al., 2004; Kažimírová et al., 2009). Bhilwade et al. (2014) who studied DNA damage in peripheral blood of Swiss mice, observed an age-dependent increase in the spontaneous MN frequency in bone marrow, in tail-moment values obtained with comet assay. Accumulation of DNA damage at older age could be due to reduced repair capacity with age and a high receptiveness to DNA damage upon external environmental exposure (Bohr et al., 1995; Walter et al., 1997).

In the present study, the MN frequency showed a significant increase with age, most likely due to an increase in acentric fragments produced by unrepaired DNA strand-breaks induced either by exposure to radiation or endogenously or by spindle disturbances resulting in chromosome lagging (Fenech et al., 2011). Gender-related differences in MN frequency could not be evaluated, as only male rats were analyzed.

Conclusion

In the present study, the effect of chronic low-dose background radiation exposure on rat lymphocytes was evaluated using CBMN assay and results showed that this exposure has no significant effect on MN frequency. This may be due to the fact that the level of radiation exposure is too low to induce significant MN. On the other hand, it is possible that damage is induced, but repaired, in rats exposed to background radiation. Studies of the DNA repair capacities and radio-adaptive responses of rats exposed to background radiation could reveal the exact mechanism at play behind the non-induction of significant MN frequency.

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