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Assessment of *In vitro* X-Ray Radiation Overexposure by Cytokinesis-Block Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBLs) in Saudi Population

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Abstract

Background: With increasing the important of ionizing radiation application in medicine, however, Ionizing radiation may be cause chromosomal aberrations as increased frequencies of micronuclei (MNi) and chromosome abnormalities. The cytokinesis block micronucleus (CBMN) assay is an established biodosimetry technique developed originally for the measurement of micronuclei exclusively in binucleated (BN) cells in peripheral blood lymphocytes

Objective: This study aimed to evaluate the relationship of DNA damage parameters; micronuclei (MNi) frequencies, binucleated cells (BNCs) and nuclear division index (NDI) of peripheral blood lymphocytes cells (PBLcs) taken from healthy donors with x-ray radiation dose rate

Materials and Methods: We performed prospective analysis on 20 peripheral blood lymphocytes samples taken from healthy volunteers. The blood samples were irradiated with single X-rays doses of 320 KeV with dose rate of 0.913 Gy/min and blood samples were exposed at the dose levels of 0, 0.5, 1, 2, 3, 4, and 5 Gy. The blood samples were then cultured for 72 h at 37°C and processed following the International Atomic Energy Agency standard procedure with slight modifications

Results and Discussion: We observed significantly increase in the average number of micronuclei with increasing radiation dose as compared with control subjects, the highest average number of MNi (400.700 ± 14.343) was found in irradiated female lymphocytes at 5Gy dose, while minimum average numbers of MNi (0.700 ± 14.343) was in non-irradiated female lymphocytes samples. The number of micronuclei in BNCs cells for healthy tended to be greater in females relative to males at lower doses of radiation (0.5- 2Gy), but this effect was not statistically significant at high doses (3–5 Gy). Average numbers of Binucleated cells and nuclear division index were significantly decreased by increasing radiation dose as compared with control groups

Conclusion: The increased number of nuclei following high radiation doses could represent a negative impact on public health especially that of workers exposed to radiation.

Keywords: Micronucleus assay; Biodosimetry; Radiation accidents; Ionizing radiation and human blood lymphocytes

Introduction

Radiation exposure due to radiotherapy or following a large-scale radiological accident in a populated area can damage the human tissues considerably [1-3]. The ability to achieve an accurate estimate of the absorbed radiation dose is critical for people exposed to significant levels of ionizing radiation, as this information can predict the health risks. Typically, rare cases of radiation exposure and limited number of potential casualty's scenario. The main focus for such isolated cases, the primary focus is on providing the most accurate dose estimate, considering details of the exposure, such as the type and quality of the radiation and the uniformity, duration, and timing of the exposure [4,5]. The ionizing radiation transferred in the living cells by the genetic material in nucleus and mitochondrial [6]. Chromosomal aberration is widely known as cytogenetic indicators used to assess radiation damage by ionizing radiation exposure for supporting the treatment of radiation casualties [7] using a biodosimetry assays as cytokinesis-block micronucleus (CBMN) and dicentric chromosome assay (DCA) [8]. The CBMN assay is a very reliable documented method to quantify chromosome breakage and loss in nucleated cells [9,10]. And *in vitro*, to assess cancer susceptibility or radiosensitivity in individuals exposed to occupational, medical, and accidentally radiation [11-13]. To study chromosomal damage *in vitro* induced by IR or chemicals using a quality dependence dosimeter, MN assay of HPBLs for radiation protection purposes according to the International Atomic Energy Agency recommendation [14-16].

In human and mammalian cells The CBMN assay used for genetic toxicology testing by assessing micronuclei (small nuclei) arise during exposure to various clastogenic agents due to mis-repaired DNA double strand breaks, during cell division small acentric chromosome fragments are not transfer to nuclei of daughter cells. MN scoring very restricted preventing confounding effects during the cell division result in a cell recognized by their binucleate (BN) appearance [9,17,18]. The *in vitro* micronucleus test is simple, useful, and applicable standard cytogenetic test for genotoxicity in different cell types [19-21]. The most useful micronucleus assay performed by adding the cytochalasin

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B in cell culture for produce binucleated cells (BNCs) [22]. In addition, scoring of micronuclei in the CBMN assay is easy and quick, making it much less labor-intensive than the DCA method and an attractive option for genetic damage assessment in population a high dose of radiation in the case of large-scale radiation accidents [17]. The main aim of this study was to assess the effect of overexposure to radiation on the peripheral blood lymphocytes cells isolated from the blood of Saudi volunteers as a reliable biomarker for measuring the emerging DNA damage, this assessment was carried out by visual scoring the micronuclei frequency, micronucleated cells and The nuclear division index in 1000 peripheral blood lymphocytes cultures of non-irradiated (control) and irradiated (0.5-5Gy) lymphocytes samples.

Material and Methods

Blood sampling

This study is a prospective study performed in six-month period between May - Oct. 2016. a total of 20 Peripheral blood samples were collected from nonsmoking and apparently healthy human volunteers had no history of exposure to radiation and did not complain from acute or chronic illness at king sultan hospital. The age range was between 25 and 35 years that consist of 10 males and 10 females. Draw aliquots of 2 mL of whole blood in heparinized vials using a vacutainer system. The study Subjects gave informed consent and the approval of a local ethics committee.

Exposure of HPBLs to X-ray radiation

In vitro, irradiated the Heparinized blood samples immediately after venipuncture using single doses of X-ray with a mean photon energy of 320 keV (filtered with 1 mm) using X-RAD 320 System (Precision X-Ray, United States) at 37°C. The dose rate was approximately 0.913 Gy/min and to obtain a calibration curve, blood samples were exposed at the dose levels of 0, 0.5, 1, 2, 3, 4, and 5 Gy. The irradiation was performed according to IAEA procedure [16], then, kept the blood samples at 37°C to allow for any chromosomal repair to take place.

Isolation and culture of lymphocyte

Blood culture and harvest according to the IAEA protocol [16,23] with slightly modified. Briefly, 0.5 ml of non-irradiated and irradiated peripheral blood samples was added to RPMI 1640 culture medium (4.5 ml) enriched with L-glutamine containing 10-15% fetal calf serum, 2% penicillin, 3% streptomycin and stimulated with 3% phytohaemagglutinin (Sigma, USA) at 37 °C. After 48 hours of culture, add 45 µl Cytochalasin B (Sigma Aldrich). After 72 hours of incubation period, centrifuging the blood sample for cell collection at 800 rpm for 5 minutes. The collected cells were suspended in 6 ml of cold hypotonic solution (0.075 M cold KCl), centrifuged at 800 rpm for 5 min. the cells were fixed in methanol: Glacial acetic acid (6:1) for 3 times.

Slide preparation and micronuclei scoring

The cytokinesis-block micronucleus test was utilized to evaluate chromosomal damages [23]. Lymphocytes fixed cells were dropped on the glass slides, air-dried and stained with 5% aqueous solution of Giemsa dye Sigma Aldrich) for 15 min. Assessment of slides was carried out using Nikon microscope with ×100 magnification to assess the MN frequency in CBMN according to the criteria proposed by (9, 16). A total of 1000 lymphocytes were examined for the Number of BN and the Number of MN cells.

Statistical analysis

The obtained data of this study was subjected to calculate the frequency, statistical description (Mean, SE) and using statistical analysis of variance (ANOVA) test and least significantly difference (LDS) was set to $P<0.05$ using SPSS statistical software V. 19.

Results

In the preliminary study, lymphocytes cultures of non-irradiated (control) and irradiated peripheral blood samples were subjected to the cytokinesis-block micronucleus assay according to standard protocol as described by Varga D [24] as a potential method to assess radiation overexposure, Visual scoring the frequencies of binucleated cells (BNCs), micronuclei (BNi) and Nuclear division index (NDI) on 1000 peripheral blood lymphocytes samples exposed to X-rays from 0.5 to 5 Gy as compared with control samples (Table 1). The (NDI) was significantly higher in non-irradiated male control lymphocytes cultures (0.918 ± 0.03, 0.89 ± 0.03) and gradually increased by increasing x-ray dose, while minim average numbers of NDI (120 ± 0.03) was in irradiated male lymphocytes with 5 Gy dose.

Study results showed that the highest average numbers of BNCs scored in 1000 non-irradiated and irradiated lymphocytes cultures were found in non-irradiated male samples (920.40 ± 30.038) and relatively decreased in accordance with increase radiation dose in both sex, while minim average numbers of BNCs (120.40 ± 30.038) was in irradiated male lymphocytes at 5Gy dose (Figure 1a). We observed that the number of MNi scored in BNCs increased proportionally with radiation dose rate (R^2=0.89; Figure 1b). The highest average number of MNi (400.700 ± 14.343) was found in irradiated female lymphocytes at 5Gy dose; while minimum average numbers of MNi (0.700 ± 14.343) was in non-irradiated female lymphocytes samples (Figure 2).

The effects of gender on DNA damage measured using micronuclei frequencies in peripheral blood lymphocytes were firstly reported by Fenech, M [25]. Our observations in this study indicated the influence of gender on the average of micronuclei scores in binucleated lymphocyte cells for healthy tended to be greater in females relative to males (Mean; 191.671 ± 5.421 and 233.443 ± 5.421) respectively as represented in

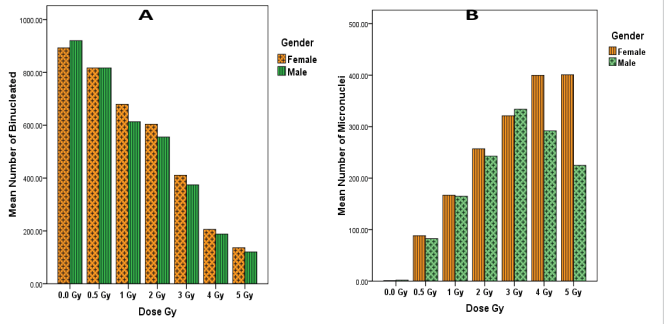

Figure 1: Mean of micronuclei (A) scored in binucleated cells (B) in 1000 non-irradiated and irradiated PBLs sample based on gender.

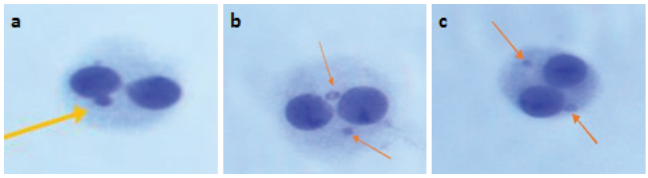

Figure 2: A typical binucleate cells with one micronucleus (a) or two micronuclei (b, c) in PBls samples exposed to x- ray and observed at 100-fold magnification..

Irradiated Dose (Gy)	Gender	BNCs (Mean ± SE)	MNi (Mean ± SE)	NDI (Mean ± SE)
Dose 0 Gy	M	920.40 ± 30.038	1.800 ± 14.343	0.918 ± 0.03
	F	893.1 ± 30.038	0.700 ± 14.343	0.89 ± 0.03
Dose 0.5 Gy	M	816.7 ± 30.038	82.500 ± 14.343	0.816 ± 0.03
	F	816.8 ± 30.038	88.100 ± 14.343	0.817 ± 0.03
Dose 1 Gy	M	613.40 ± 30.038	164.600 ± 14.343	0.613 ± 0.03
	F	679.5 ± 30.038	166.900 ± 14.343	0.679 ± 0.03
Dose 2 Gy	M	555.4 ± 30.038	242.500 ± 14.343	0.555 ± 0.03
	F	604.0 ± 30.038	256.900 ± 14.343	0.604 ± 0.03
Dose 3 Gy	M	374.4 ± 30.038	333.800 ± 14.343	0.374 ± 0.03
	F	410.80 ± 30.038	321.100 ± 14.343	0.411 ± 0.03
Dose 4 Gy	M	188.299 ± 30.038	291.800 ± 14.343	0.188 ± 0.03
	F	205.60 ± 30.038	399.700 ± 14.343	0.206 ± 0.03
Dose 5 Gy	M	120.40 ± 30.038	224.700 ± 14.343	0.120 ± 0.03
	F	136.60 ± 30.038	400.700 ± 14.343	0.137 ± 0.03
Based on observed means, the mean difference among non-irradiated and irradiated PBLs sample is significant at the 0.05 level				

Table 1: The average values of binucleated cells, micronuclei and division index scored in 1000 lymphocyte cells of male and female after X-ray exposure.

(Figure 3). However, this was only true at lower doses of radiation (0.5–2 Gy), but at high doses (3–5 Gy), there was no statistically significant difference in MNi percentage between groups 4 Gy4: female (399.7) and male (291.8) and Gy5: female (400.7) and male (224.7) (Figure 1b).

Discussion

The present study investigated the effects of X-ray irradiation on human peripheral blood lymphocytes (HPBLs) by evaluating DNA damage using CBMN assay, as potential tool for assessing cytogenetic damage induced by radiation [26,27]. In general, Parameters of BNCs, MNi and NDI were significantly, and dose dependently changed after irradiation by 1 – 5 Gy as compared with control subjects. On the other hand, cellular proliferation decreased with increasing radiation dose, in agreement with previous work on HPBL irradiated with X-rays [28]. We also investigated differences in DNA damage induced by X-rays by measuring MN in binucleated cells. The mean baseline frequency of MN in non-irradiated lymphocytes was similar to that of previous reports and was within the expected range [8]. A decrease in cellular proliferation and increased cytogenetic damage were showed at higher doses, thereby increasing cell death [29]. The biological responses of irradiated cells also depend on the dose rate [25]. Specifically, a decreased dose rate can result in decreased micronuclei formation in HPBL, possibly due to efficient repair [30,31].

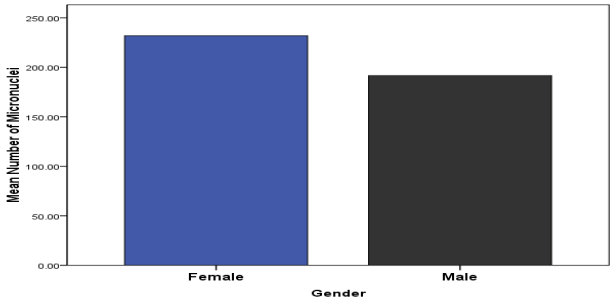

Figure 3: Frequencies of micronuclei based on gender.

NDI is a measure of cytostasis and cell death (cytotoxicity) as a marker of cell proliferation. The rationale behind NDI is cells with greater chromosomal damage are less likely to enter cell division or cell death occurs before cell division [32]. Our findings showed decreased BNCS and NDI in the x-ray irradiated samples in comparison to the controls subjects. If all viable cells complete one division during the cytokinesis-block phase the binucleated cells will be formed and they will contain more than two nuclei If some viable cells complete more than one nuclear division [32,33]. Analysis of nuclear division index revealed significant differences in lymphocyte proliferation rate upon irradiation; this agrees with an earlier study reporting that DNA damage response was altered by irradiation [18].

A reduction in the dose rate may favor the arrest of cells with DNA damage in the G1 and G2 phases over cell, and the transducer protein kinase ataxia-telangiectasia mutated (ATM) preventing the cells from replicating damaged DNA and the DNA repaired with time, in contrast to the induction of apoptosis of damage cells that occurs at a high dose rate [30]. Micronuclei are formed because of chromosome either breaks or fail to engage with the mitotic spindle fibers.

In 1985, Fenech established the CBMN assay in HPBL (9) this cytogenetic dosimetry assay is no requirement for metaphase cells, speed and ease of analysis, identification the cells completed one nuclear division [34,35]. In an emergency, radiation dose estimates should be provided as soon as possible with sufficient accuracy to support clinical decision-making. Cell-cycle progression delayed in cells that exposed to the radiation and produce s micronuclei and quickly died this can result in fewer cells reaching mitosis. The CMBN assay requires 72 hours of culture time, after which cell processing can occur followed by slide preparation. MN scoring is easier with this assay than the DCA method [36]. The biodosimetryt recommended that scoring 1,000 binuclear cells [37] in approximately 2 hours, recently Lindholm and colleagues demonstrated that scoring of 200 binuclear cells in about 15 minutes was sufficient to identify radiation doses of >1 Gy [38].

There are some drawbacks to the CBMN assay; however, new improvements have made the assay more adapted to radiation exposure [39] as automated and high-throughput scoring analysis [40,41]. A limited dose range of CBMN assay (0.3–5 Gy), although it has been suggested that the modifications to the method may be attained a range of 0.1–15 Gy [8,36,39,41-45]. Experiments proven that CBMN assay is reliable for up to 6 months after exposure (46), but with a correction factor the time can be extended to approximately 1 year [46-48].

Previous study examines the micronuclei frequency in HPBL, the results showed a significant increase in the number of micronuclei in irradiated blood samples. Other studies have proved that the yield of dicentric chromosomes and micronuclei is increased by the prolonged culture of irradiated lymphocytes cells with PHA at different time intervals [49,50]. Comparative outcomes have been reported that levels chromosomal aberrations result from delay of lymphocytes stimulation cells due to radiation exposure that were elevated compared to lymphocytes stimulated immediately [51]. Furthermore, in stimulated lymphocytes A critical decrease in micronuclei frequency by checkpoint activation result in cell-cycle arrest or by repair of DNA damage induced by the radiation exposure at twenty hours post-irradiation, compared to two or six hours post-irradiation [52].

Conclusion

As a result of this study we can conclude, accumulation of genetic damage is detectable in peripheral lymphocytes of healthy individuals

exposed to x-ray radiation. The MN frequency increases and affected by gender while the BNCs and NDI decreases with increase the dose rate. Our results suggest that Establishing a laboratory which competent enough to perform cytogenetic analysis for biodosimetry is very important in Saudi Arabia for measuring micronuclei in BNCs cells as best biomarker to assess radiation damage evaluated by CBMN assay.

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Conflict of Interest

Between me and co-authors, there is no any indirect or direct financial interest we have in the subject matter of a submitted manuscript, or any other potential conflict of interest. Also, thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response.

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