Iran. J. Radiat. Res., 2011; 8(4): 231-236

Dose estimation with the calibration of dose-response curve of micronucleus in human peripheral lymphocytes induced by 50MeV proton beams

Y.J. Go[1], J.H. Shin[1], K.S. Jeong[1], S.J. Park[1], M.H. Lee[1], D.M. Kwak[1], O.D. Kwon[1], S.H. Kim[2], S.Y. Ryu[3], C.H. Kim[4], E.J. Kim[4], Ch.M. Kang[4*], TH. Kim[1*]

[1]College of Veterinary Medicine, Kyungpook National University, Korea
[2]College of Veterinary Medicine, Chonnam National University, Korea
[3]College of Veterinary Medicine, Chungnam National University, Korea
[4]Korea Institute of Radiological and Medical Sciences, 1370 Sangyeok-Dong, Buk-ku, Taegu, 702-701, Korea

Background: The purpose of this paper is to establish an easy and reliable biodosimeter protocol to evaluate the biological effects of proton beams. ***Materials and Methods:*** Human peripheral blood lymphocytes were irradiated using proton beams (LET: 34.6 keV μm^{-1}), and the chromosome aberrations induced were analyzed using cytokinesis-blocked (CB) micronucleus (MN) assay. To determine the efficiency of MN assay in estimating the doses received by 50MeV proton beams and to monitor predicted dose of victims in accidental exposure, here we have evaluated the performance of MN analysis in a simulated situation after exposure with proton beams. Peripheral lymphocytes were irradiated by 50MeV proton beams up to 6Gy and analyzed by Giemsa staining of CB MN assay. ***Results:*** The detected MN was found to be a significant dose-effect curve in the manner of dose-dependent increase after exposure with proton beams *in vitro*. When plotting on a linear scale against radiation dose, the line of best fit was $Y=0.004+(1.882x10^{-2}\pm9.701x10^{-5})D+(1.43x10^{-3}\pm1.571x10^{-5})D^2$. Our results show a trend towards increase of the number of MN with increasing dose. It was linear-quadratic and has a significant relationship between the frequencies of MN and dose (R^2= 0.9996). The number of MN in lymphocyte that was observed in control group is 5.202±0.04/cell. ***Conclusion:*** Hence, this simple protocol will be particularly useful for helping physicians to decide medical therapy for the initial treatment of victims with rapid and precise dose estimation after accidental radiation exposure. Also it has potential for use as a valuable biomarker to evaluate the biological effectiveness for cancer therapy with proton beams. **Iran. J. Radiat. Res., 2011; 8(4): 231-236**

Keywords: *Human peripheral lymphocyte, biodosimetry, proton beams, dose estimation, triage.*

INTRODUCTION

We have studied to develop the useful biodosimetry through the most radiosensitive biomarkers for two decades. These studies have been tried to elucidate more chromosome aberrations with respect to its biological effects on human lymphocytes that are highly susceptible to ionizing radiation. Especially, there are problems involving the exposure with proton beams. Although the biological effects of proton beams are of considerable concern in the National Aeronautics and Space Administration because proton beams in deep-space radiation is more than 80% of all ionizing radiation, dose estimation of individuals to predict their cytogenetic damage of radiosensitive organs has been studying due to the lack of sufficient data [1, 2].

Characteristics of proton beams are generally high-LET with an energy deposition peak (Bragg peak) at the end of their tracks and a higher relative biological effectiveness (RBE) within the peak region. These aspects make proton attractive in various scientific disciplines, especially in nuclear industries and radiotherapy.

Corresponding authors:
Prof. Tae-Hwan Kim Kim and Chang-Mo Kang, College of Veterinary Medicine, Kyungpook National University, Daegu city, 702-701, Republic of Korea.
Fax: *+82 53 950 5977*
E-mail: *thkim56@knu.ac.kr, kangcm@kcch.re.kr*

Numerous researchers have reported severe depression of immune system to capable to injury host defense mechanism after radiotherapy with conventional radiation protocols including gamma rays, neutron and electron beams. The physical characteristics of proton beams give it an advantage over X-rays and electron beams therapy of cancer patients because the maximum dose can be delivered to the tumor by Bragg peak, while the absorbed dose in normal tissues around the target tumors is minimized. Since it is possible to safely deliver a higher dose to the desired target tissues, the possibility for cancer treatment with proton beams is increased [3, 4].

However, although many publications of the biological effects of X-rays and gamma rays have been reported, very few have been documented the biological effects of proton beams delivered to normal tissues in case of radiotherapy until now [5, 6]. A better understanding of biological interactions that occur after the exposure with proton beams is needed in order to optimize therapeutic regimens and facilitate development of strategies that decrease radiation-induced cytogenetic damages and its side effects. Furthermore, the biological properties of proton beams have been reported identical or very similar to those of gamma rays, especially RBE [7]. MNs are regarded as being the most sensitive biomarker of radiation-induced genetic damages [8, 9]. Scoring of MNs in human lymphocytes is often used to determine cytogenetic damages to radiation exposures, because the CB-MN protocol is simple and well-established [10-12]. Therefore, the differential process of damaged cells with the overexposure to high-LET radiation may lead to an underestimation of the absorbed dose of patients by dilution of the cytogenetic damage in assessing the dose estimation and the understanding of biological effects in the radiosensitive target organ through peripheral lymphocytes, which is conventionally confined to first-division metaphase lymphocytes collected at 72h post-irradiation [13, 14].

The present study quantified human peripheral lymphocytes after the exposure with proton beams at varying doses *in vitro* [15]. We used ^{137}Cs gamma rays as a reference radiation, which provide more relevance to clinical practice. Dose-response relationships of proton beams were established by analyzing MN frequencies in human peripheral lymphocytes *in vitro* using Giemsa staining of CB MN assay [16-18].

MATERIALS AND METHODS

Cell culture

Human peripheral blood from 20 healthy volunteers aged between 21 years and 50 years with no history of exposure to mutagenic agents including radiotherapy were obtained by the venipuncture. In all cases, peripheral blood lymphocytes were separated from whole blood cells on Ficoll-Hypaque gradients, washed twice in Hank's balanced salt solution and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) containing Hepes buffer, 15 % heat inactivated fetal calf serum, L-glutamine and antibiotics. Lymphocytes were cultured in 15ml Falcon test tubes (Corning, No. 25820, NY) at a concentration of 5×10^5 cells/ml. An optimum concentration of phytohemagglutinin (PHA, 5mg/ml, Sigma, St. Louis, Mo) was used to stimulate the lymphocytes to transform and divide in culture. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 [19].

Irradiation condition

Control group of the non-irradiated samples served as a reference for determining the spontaneous MN frequencies. For the equation of the dose-effect relationship, all whole blood samples were irradiated with 1, 2, 4 and 6Gy of proton beams, respectively. The dose rate was 211cGy/min. The doses were measured with a Capintec PR-06C farmer type chamber and a Capintec 192 electrometer (Capintec, U.S.A.) [19, 21, 22].

Cytokinesis-block methods

Cyt-B (Aldrich Chemical Co., West Saint Paul) was made up as a stock solution in dimethyl sulfoxide at a concentration of 2 mg/ml, divided into small portions and stored at -70°C. The stocked solution of Cyt-B was thawed, diluted in medium and added 44 h after commencement of the culture at a concentration of 3.0µg/ml. After an incubation period of 72 h, the cells were collected by centrifugation and resuspended in a mixture of methanol: glacial acetic acid (3:1). The fixed cells were transferred to a slide, air-dried and stained with 10% Giemsa for 10 min [18].

Scoring of micronuclei and data analysis

The MNs were scored in 1000 binucleated CB cells using a 400x magnification. All analyses were performed using a Graph PAD in Plot computer program (GPIP, Graph PAD Software Inc., San Diego) and Excel program [19].

RESULTS

Induction kinetics of MN in human peripheral lymphocyte after the exposure with proton beams

A preliminary investigation was done to determine the optimal concentration of Cyt-B for accumulating CB cells. The optimal Cyt-B concentration appeared to be 3.0 mg/ml and was used throughout the experiments in this study.

To evaluate the dose-response equation after the exposure with proton beams, the frequencies of the induced MN was obtained by subtraction of the number of MN scored in the non-irradiated control group from the total numbers of those cells in the irradiated groups. The frequencies of MNs on peripheral lymphocytes depending on age and sex in Korean population were observed in table 1. The morphological findings of the induced MN were typical in lymphocytes, as shown in figure 1.

The average numbers of MNs induced by proton beams, obtained by pooling the LM data of 20 subjects, are presented as a function of radiation dose and the error bars represent standard deviations within the studied population. The frequencies of MNs increased with the dose. The spontaneous MN frequencies in lymphocytes of the non-irradiated control groups showed no significant difference between individuals. The baseline number of MN per cell in non-irradiated control group was low, being 5.202±0.04.

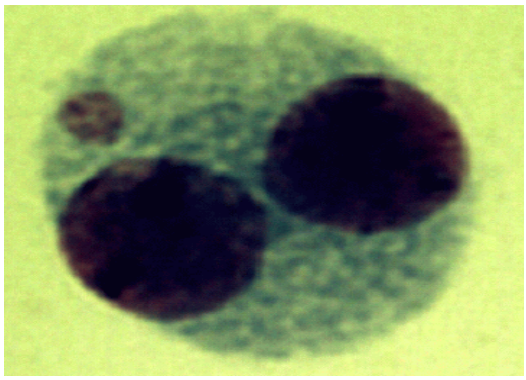

Figure 1. Finding of the induced MN stained H&E after the exposure with proton beams.

Table 1. Frequencies of micronuclei on peripheral lymphocytes were observed depending on age and sex in Korean population.

Age groups	Average number of micronuleus	
	Female (n)	Male (n)
20-29	4.05±0.07 (200)	2.0±0.5 (160)
30-39	6.12±2.02 (90)	4.19±1.09 (80)
40-49	13.42±1.3 (60)	7.19±1.02 (65)
50-59	16.21±1.7 (67)	13.45±1.08 (70)
60-	20.52±2.6 (55)	16.6±2.05 (65)

Dose-response relationship

To evaluate the equation of dose-response curves, the number of MN per cell was examined at the different doses, and dose-response curve of the induced MN was obtained by fitting the linear-quadratic model $y=a+bD+cD^2$, where y is the yield of MN/cell, a is the spontaneous yield, b is the coefficient of the one-track component, c is the coefficient of the two-track component, and D is the dose in Gy. When plotting on a linear scale against radiation dose, the line of the best fit was $Y=0.004+(1.882\times10^{-2}\pm9.701\times10^{-5})D+(1.43\times10^{-3}\pm1.571\times10^{5})D^2$ (R^2= 0.9996) after the exposure with proton beams (figure 2). There was a significant relationship between the frequency of MN and dose. The dose-response curves were linear-quadratic. These data show trends towards increasing MN numbers with increasing dose.

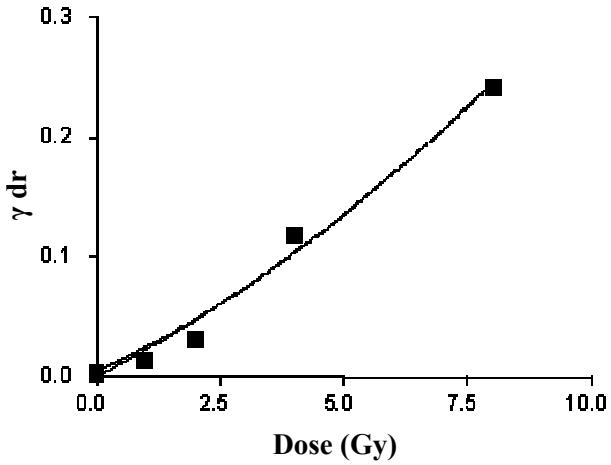

Figure 2. Dose-response curves of the induced MN in human peripheral lymphocytes up to 6Gy after the exposure with proton beams.

DISCUSSION

At present, there are few biological indicators that can be used for monitoring dose limits of medical and occupational radiation exposure [4, 9]. The complexity of dose estimation and their association with radiation exposure is various in the biological monitoring of cytogenetic damages. Until now, this places chromosomal aberrations assay a reliable biomarkers [7, 8]. Cytogenetic indicators as whole can be helpful in evaluating cellular damages in *in vivo* and *in vitro* mechanisms underlying DNA damage induced by ionizing radiation. Generally, radiobiological monitoring of humans has relied heavily on cytogenetic indicators such as unstable chromosomal aberrations. Also other cytogenetic indicators such as MN in human lymphocytes have been used in the field of biological dosimetry as well as in predicting cancer risk [11, 15, 17]. Our use of MN for evaluating the biological effects of cancer patients after the radiotherapy with our developed proton beams, mentioned above, has been helpful in confirming dose estimation and in predicting the extent of DNA damages in different scenarios, namely after an environmental, occupational, accidental or medical exposure although average frequencies of MNs is different depending on life-style, sex, age and species just like my own date shown in table 1. Our particular interest is the follow-up study of cancer patients treated with our developed proton beams.

Recently, we have studied to find novel cytogenetic indicators to evaluate the absorbed dose of victims and radiotherapeutic patients after accidental, occupational, environmental or therapeutic exposure with ionizing radiation for 20 years. Of our results lymphocytes from all subjects revealed an increase of the frequencies of cytogenetic parameters such chromosome aberrations, MN, comet assay, DNA strand breaks and apoptosis induction assay [21, 22]. Dose-response relationships of MN in our studies are similar, although lower than those for chromosome aberrations, since not all acentric fragments give rise to MNs. The dose-response curves presented in Figure 2 indicate that MN was significantly sensitive than that of expected process in our developed proton radiation. In human lymphocytes, the persistence of cytogenetic damages over time depends on various factors, including the type of biomarkers and the severity of the outcome to the cell, which can induce mitosis-linked cell death

and apoptosis, or renewal of the lymphocyte population [23].

Mechanistic knowledge of DNA and cell damage by high LET radiation, such as heavy ions, is less extensive compared to that of low LET radiation. Since proton beams are capable of traversing a fraction of the limited cell volume through break peaks, they are very effective in producing a high density of localized lesions. So DNA lesions produced by proton beams are characterized by clustering, inducing DSBs, which are difficult to repair. Complex chromosome rearrangements leading to the enhanced cell death lead to a greater biological effectiveness per unit dose for high LET radiation. One instance of high LET radiation for applying in the treatment of malignant tumors is the radiotherapy of our developed proton beams that depend on the induced length of break peaks. These malignant cells reveal a characteristic pattern with the presence of multi-micronucleated cells. These results are consistent with the formation of multiple damaged sites on the DNA molecule [24-26]. Therefore, the pattern of cytogenetic damage may be used to evaluate biological effectiveness of cancer cells after the radiotherapy with our developed proton beams. It is important to develop simple and reliable techniques for evaluating radiation-induced genetic alteration of malignant cells after the radiotherapy with our developed proton beams [1, 8, 11-15].

Our results showed more reproducible, dose-related and quantifiable up to 6Gy in our developed proton beams. With this approach it would be possible to detect easily biological effects of doses in case of the radiotherapy with proton beams and the screening of cancer patients as one of the most sensitive radiobiological endpoints. Here, since our data present that MN frequencies increased with increasing radiation doses in statistical distribution, it may be a simple and reliable biological dosimeter for the evaluation of radiobiological effectiveness of cancer patients after the radiotherapy with proton beams.

ACKNOWLEGMENTS

This research was supported by Kyungpook National University Research Fund.

REFERENCES

1. Thierens H and Vral A (2009) The micronucleus assay in radiation accidents. *Ann Ist Super Sanita,* ***45:****260-264.*
2. Chao NJ (2007) Accidental or intentional exposure to ionizing radiation: Biodosimetry and treatment. *Exerimental Hepatology,* ***35****: 24-27.*
3. Amundson SA, Bittner M, Melzer P, Trent J, Fornace AJ Jr (2001) Biological indicators for the identification of ionizing radiation exposure in humans. *Exp Rev Mol Diagnostics* ***1****: 211-219.*
4. TH Kim, SH Kim, JH Kim, YS Lee, CK Cho, SY Choi, SH Park, SY Yoo (1996) Measurement of apoptotic fragments in growing hair follicles following gamma-ray irradiation in mice. *Anticancer Res*, ***16:*** *189-192.*
5. SH Kim, TH Kim, IY Chung, CK Cho, KH Ko, SY Yoo (1992) Radiation-induced chromosome aberration in human peripheral blood lymphocytes *in-vitro*: RBE study with neutron and ^{60}Co g-rays. *Korean J Vet Res*, ***17:*** *21-30.*
6. Amundson SA, Bittner M, Melzer P, Trent J, Fornace AJ Jr (2003) Functional genomics as a window on radiation stress signaling. *Oncogens*, ***22:*** *5828-5833.*
7. Blakely WF, Carr Z, Chu MC, Dayal-Drager R, Fujimoto K, Hopmeir M, Kulka U, Lillis-Hearne P, Livingston GK, Lloyd DC, Mazyk N, Perez Mdel R, Romm H, Takashima Y, Voison P, Wilkins RC, Yoshida MA (2009) 1st consultation on the development of a global biodosimetry laboratories network for radiation emergencies(BioDoseNet). *Radiat Res*, ***171:*** *127-139.*
8. Das B, Karuppasamy CV (2009) Spontaneous frequency of micronuclei among the newborns from high level natural radiation areas of Kerala in the southwest coast of India. *Int J Radiat Biol*, ***85:****272-280.*
9. Fench M and Morley AA (1989) Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss. *Mutagenesis,* ***4:*** *98-104.*
10. Demidenko E, Williams BB, Swarz HM (2009) Radiation dose prediction using data on time to emesis in the case of nuclear terrorism. *Radiat Res*, ***171:*** *310-319.*
11. Almassy Z, Kanyar B, Koteles GJ (1986) Frequency of micronuclei in X-irradiated human lymphocytes. *Int J Radiat Biol*, ***49:*** *719-723.*
12. Savage JRK (1988) A comment on the quantitative relationship between micronuclei and chromosomal aberrations. *Mutat Res*, ***207:*** *141-146.*
13. Muller WU and Sreffer C (1994) Micronucleus assay. In: Obe G, ed. Advances in mutagenesis research. Berlin: Springer. ***4:****1-133.*
14. Catena C, Conti D, Del Nero A, A, Righi E (1992) Interindividual differences in radiation response shown by an *in-vitro* micronucleus assay: effects of 3-aminobenzamide on X-ray treatment. *Int J Radiat Biol*, ***62:*** *687-694.*

15. Thierens H, Vral A, De Ridder L (1991) Biological dosimetry using the micronucleus assay for lymphocytes: interindividual differences in does-response. *Health Phys*, ***61:*** *623-630.*
16. Straume T, Lucas JN, Tucker JD, Bigbee WL, Langlois RG (1992) ***62:*** *122-130.*Biodosimetry for a radiation worker using multiple assays. *Health Phys*,
17. Gantenberg HW, Wuttke K, Streffer C, Müller WU (1991) Micronuclei in human lymphocytes irradiated *in-vitro* or *in-vivo*. *Radiat Res*, ***128:*** *276-281.*
18. Littlefield LG, Sayer AM, Frome EL (1989) Comparison of dose-response parameters for radiation-induced acentric fragments and micronuclei observed in cytokinesis-arrested lymphocytes. *Mutagenesis*, ***4:*** *265-270.*
19. Prosser JS, Moquet JE, Lloyd DC, Edwards AA (1988) Radiation induction of micronuclei in human lymphocytes. *Mutat Res*, ***199:*** *37-45.*
20. Evans HJ (1988) Mutation cytogenetics: past, present and future. *Mutat Res*, ***204:*** *355-363.*
21. SH Kim, TH Kim, SY Yoo, KH Ko, HG Yun, SY Choi (1993) Frequency of micronuclei in lymphocytes following gamma and fast-neutron irradiations. *Anticancer Res*, ***13:*** *1587-1592.*
22. Bauchinger M, Schmid E, Rimpl G *et al.* (1975) Chromosome aberrations in human lymphocytes after irradiation with 15.0-MeV neutrons *in-vitro*. I. Dose-response relation and RBE. *Mutat Res*, ***27:*** *103-109.*
23. Lloyd DC, Purrott RJ, Dolphin GW, Edwards AA (1976) Chromosome aberrations induced in human lymphocytes by neutron irradiation. *Int J Radiat Biol*, ***29:*** *169-182.*
24. Huber R, Schraube H, Nahrstedt U, Braselmann H, Bauchinger M (1994) Dose-response relationships of micronuclei in human lymphocytes induced by fission neutrons and by low LET radiations. *Mutat Res*, ***306:*** *135-141.*
25. Vral A, Verhaegen F, Thierens H, De Ridder L (1994) Micronuclei induced by fast neutrons versus ^{60}Co gamma-rays in human peripheral blood lymphocytes. *Int J Radiat Biol*, ***65:*** *321-328.*
26. Ono K, Nagata Y, Akuta K, Abe M, Ando K, Koike S (1990) Frequency of micronuclei in hepatocytes following X and fast neutron irradiations – An analysis by a linear-quadratic model. *Radiat Res*, ***123:*** *345-347.*