Frequency of Micronuclei in Lymphocytes Following Gamma and Fast-neutron Irradiations

Sung-Ho Kim, Ph.D.,* Chül-Koo Cho, M.D., Tae-Hwan Kim, Ph.D.*
In-Yong Chung, M.S.,* Seong-Yul Yoo, M.D.
Kyoung-Hwan Koh, M.D. and Hyong-Geun Yun, M.D.

Department of Radiotherapy and Laboratory of Radiation Medicine*, Korea Cancer Center Hospital, Seoul, Korea

The dose response of the number of micronuclei in cytokinesis-blocked (CB) lymphocytes after *in vitro* irradiation with γ-rays and neutrons in the 5 dose ranges was studied for a heterogeneous population of 4 donors. One thousand binucleated cells were systematically scored for micronuclei. Measurements performed after irradiation showed a dose-dependent increase in micronuclei (MN) frequency in each of the donors studied. The dose-response curves were analyzed by a linear-quadratic model, frequencies per 1000 CB cells were (0.31 ± 0.049) D+ (0.0022 ± 0.0002) $D^2 + (13.19 \pm 1.854)$ $(r^2 = 1.000, X^2 = 0.7074, p = 0.95)$ following γ irradiation, and (0.99 ± 0.528) D+(0.0093 ± 0.0047) $D^2 + (13.31 \pm 7.309)$ $(r^2 = 0.996, X^2 = 7.6834, p = 0.11)$ following neutrons irradiation (D is irradiation dose in cGy). The relative biological effectiveness (RBE) of neutrons compared with γ-rays was estimated by best fitting linear-quadratic model. In the micronuclei frequency between 0.05 and 0.8 per cell, the RBE of neutrons was 2.37 ± 0.17. Since the MN assay is simple and rapid, it may be a good tool for evaluating the γ-ray and neutron response.

Key Words: Micronuclei/Gamma and neutron/Biological dosimetry/Cytokinesis blocked cell/ Relative biological effectiveness

INTRODUCTION

Measurement of radiation response by simple and informative techniques would be of great value in studying genetic risk following occupational, therapeutic or accidental exposure to radiation. Biological dosimetry has a number of applications[1]. The most obvious one is in case of radiation accidents with a lack of physical dosimetry. Sometimes physical dosimetric methods must be supplemented by biological assays, for example, after partial-body exposure with the physical dosimeter outside the radiation field.

One of the biological methods adopted for dosimetry purposes, cytogenetic analysis has been the most popular one[1,2]. The occurence of chromosome aberrations in peripheral blood lymphocytes (PBLs) has been used. Although this is a sensitive method for dose estimation, it is laborious. An alternative and simple cytogenetic technique is the measurement of MN frequency in cultured human lymphocytes[3]. Compared to the classical cytogenetic methods for evaluating chromosomal damage[4], the MN assay for PBLs is relatively simple and allows a rapid scoring of a large number of cells by personnel not specially trained for chromosomal analysis[1].

The present study was performed to study micronucleus induction in human PBLs treated with γ-rays or neutrons and to determie the RBE of neutrons.

MATERIALS AND METHODS

1. Cell Culture

Blood was obtained from healthy donors aged between 18 years and 33 years. PBLs seperated from whole blood were placed on Ficoll-Hypaque gradients, washed twice in Hank's balanced salt solution and resuspended in RPMI 1640 medium (GIBCO, Grand Island, NY) containing Hepes buffer, 15% heat inactivated foetal calf serum, L-glutamine and antibiotics. The lymphocytes were cultured in multi-well tissue culture plates (Corning, No. 25820, NY) at concentration of 5×10^5 cells/ml. An optimum concentration of phytohaemagglutinin (PHA, 5 μ/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.
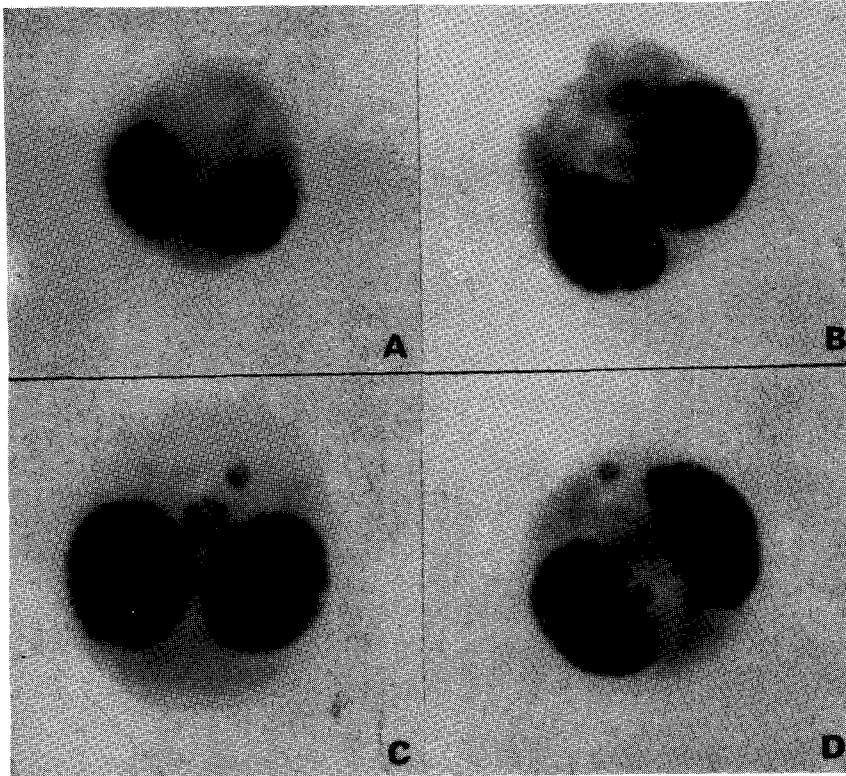
Fig. 1. Cytochalasin blocked cells without a MN (A), with 1 MN (B), 2 MN (C), and 3 MN (D).

2. Irradiation

One sample was served as a control for determining the spontaneous MN frequency. The others were irradiated with 124.8, 186.8, 280.4 and 395.6 cGy of ^{60}Co γ-rays (Theratron-780 teletherapy unit) at a rate of 211 cGy/min or 52.9, 75.6, 106.2 and 149.1 cGy of neutrons at a rate of 30 cGy/min generated by cyclotron (MC-50, Scanditronix) in a 37°C water bath, respectively. The doses were measured with Capintec PR-06C farmer type chamber and Capintec 192 electrometer (Capintec, U.S.A).

3. Cytokinesis-block Method

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

4. Scoring of Micronuclei

The MN were scored in 1000 binucleated CB cells using a 400 X magnification. For the identification of MN published criteria were applied[5]. Examples of CB cells with different numbers of MN are shown in Figure. 1. All analyses were performed using a Graph PAD In Plot computer program (GPIP, Graph PAD Software Inc. San Diego) and an AT-type personal computer.

RESULTS

The data obtained in the dose-response study for the four donors are summarized in Table 1, 2. The MN frequency in unexposed lymphocytes was not significantly different from donor to donor. The baseline number of MN per CB cell in unirradiated lymphocytes was very low as being 0.013±0.0002 (Mean±SE, Table 3). Figure 2, 3 show the results

Table 1. Micronuclei (MN) Per 1000 Cytokinesis Blocked (CB) Cells for the Individual Donors after γ-rays Exposure

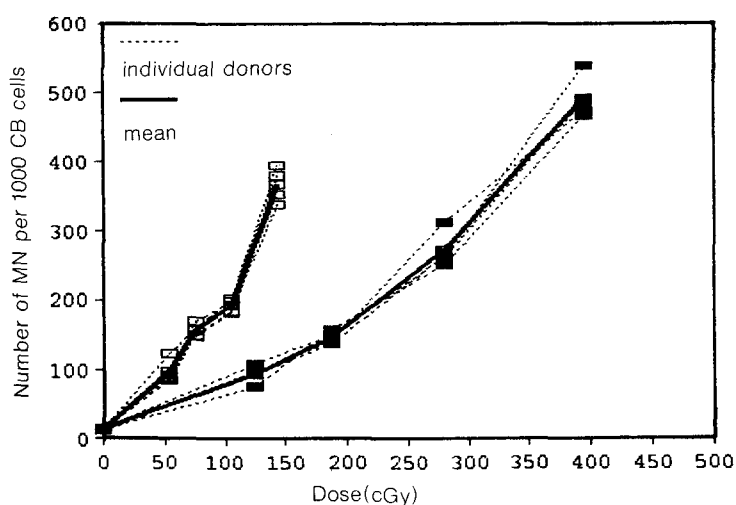| Dose (cGy)	Number of cells without MN	Frequency distribution of the number within one CB cell							Total number of MN
		1	2	3	4	5	6	7	
Donor 1: Male, 29y									
0	989	10	1						12
124.8	932	61	7						75
186.8	866	115	18			1			156
280.4	799	159	35	7					250
395.6	676	210	91	19	4				465
Donor 2: Male, 28y									
0	990	8	2						12
124.8	920	69	10	1					92
186.8	887	113	8	1	1				136
280.4	792	166	33	6	2	1			263
395.6	639	241	81	27	8	3		1	538
Donor 3: Male, 18y									
0	989	9	2						13
124.8	910	76	12	2					106
186.8	864	125	9	2					149
280.4	787	174	29	10					262
395.6	661	220	94	20	5				488
Donor 4: Male, 33y									
0	987	12	1						14
124.8	914	79	5	2					95
186.8	878	107	13	2					139
280.4	755	189	44	12					313
395.6	688	185	100	23	3			1	473

Fig. 2. The dose-response relationship of micronuclei in binucleated lymphocytes follwing treatment with γ-rays (■) or neutrons (□).

Table 2. Micronuclei (MN) Per 1000 Cytokinesis Blocked (CB) Cells for the Individual Donors after Neutrons Exposure

| Dose (cGy)	Number of cells without MN	Frequency distribution of the number within one CB cell							Total number of MN
		1	2	3	4	5	6	7	
Donor 1: Male, 29y									
0	989	10	1						12
52.9	928	62	9	1					83
75.6	885	89	21	3	1	1			149
106.2	847	128	23	3					183
143.1	754	170	61	14	1				338
Donor 2: Male, 28y									
0	990	8	2						12
52.9	922	69	6	3					90
75.6	872	110	17	1					147
106.2	858	109	27	6					181
143.1	747	198	49	20	6				380
Donor 3: Male, 18y									
0	989	9	2						13
52.9	901	82	12	5					121
75.6	864	117	16	3					158
106.2	840	125	29	6					201
143.1	752	166	61	15	5				353
Donor 4: Male, 33y									
0	987	12	1						14
52.9	925	65	8	2					87
75.6	856	123	17	4					169
106.2	839	129	27	5					198
143.1	720	193	64	18	5				395
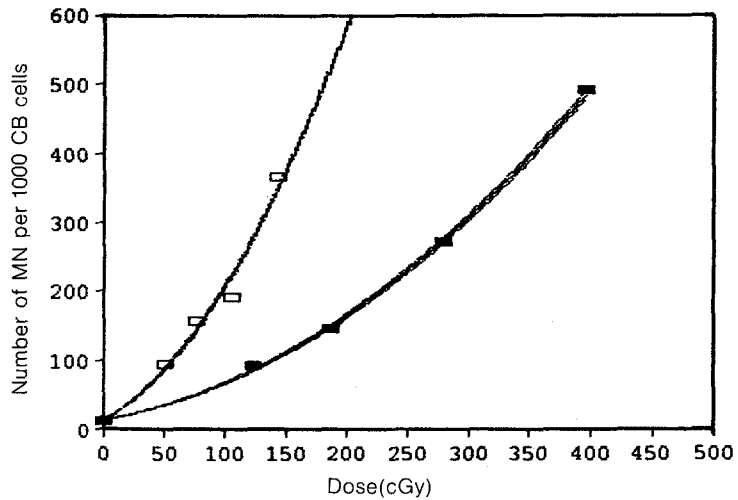
Fig. 3. Dose-response for γ-rays (■) and neutrons (□) induced micronuclei. The solid and dashed lines represent the results of a linear-quadratic fit through the data indicated in the figure.

for individual donors, the curves obtained from the pooled data of donors and best fitting linear-quadratic curves of γ-rays and neutrons, respectively. There was a significant relationship between the frequency of induced MN and dose of γ-rays ($r^2=1.000$, $\chi^2=0.7074$, $p=0.95$) and neutrons ($r^2=0.996$, $\chi^2=7.6834$, $p=0.11$). When analysed by linear-quadratic model the line of best fit was:

γ-ray: $y=(0.31\pm0.049)$ D$+(0.0022\pm0.0002)$ D^2 $+(13.19\pm1.854)$

neutron: $y=(0.99\pm0.528)$ D$+(0.0093\pm0.0047)$ D$^2+(13.31\pm7.309)$

Where y=number of MN/1000 CB cells and D= irradiation dose in cGy.

In order to determine the RBE of neutrons compared with γ-rays, the equation of $y=aD+bD^2+c$ was transformed as

$$D=\frac{[-a\pm\sqrt{a^2-4b\,(c-y)}]}{2b}$$

The RBE of neutrons for lymphocytes was obtained from this equation. In the MN frequency between 0.05 and 0.8 per cell, the RBE of neutrons was 2.37 ± 0.17 (Table 4).

DISCUSSION

The mutagenic and carcinogenic risk associated with exposure to ionizing radiation has stimulated considerable interest in measuring genetic alteration in human cells. Analysis of MN in lymphocytes is a simpler and faster method for measuring chromosome damage than any other methods. However, MN originates from acentric fragments or whole chromosomes, and this provides a measure of both chromosome breakage and loss, which is a somewhat different spectrum of damge from that obtained by chromosome analysis. Enumeration of MN in CB cells allows chromosome damage to be analysed in lymphocytes which have divided only once. This can be achieved in metaphase analysis only if bromodeoxyuridine uptake and differential staining is included in the protocol, to distinguish between first and second division metaphases. The CB method has already been shown to be simple, reliable and, above all, very sensitive[6~9] as a result of the statistical power afforded by the high scoring rate achievable (usually 1000 CB cells/30 min). The technique does not require highly specialized staff and should, therefore, be readily implemented for routine dosimetry[10~13]. Furthermore, automated scoring of MN should be relatively simpler than automated metaphase analysis and image analysis

Table 3. Frequency of Micronclei in Binucleated Lymphocytes Following Treatment with γ-rays or Neutrons

Dose (cGy)	Micronuclei per cell (M$\pm$SE)
γ-ray	
0	0.013$\pm$0.0002
124.8	0.092$\pm$0.0032
186.8	0.145$\pm$0.0023
280.4	0.272$\pm$0.0070
395.6	0.491$\pm$0.0082
Neutron	
0	0.013$\pm$0.0002
52.9	0.095$\pm$0.0044
75.6	0.156$\pm$0.0025
106.2	0.191$\pm$0.0026
143.1	0.367$\pm$0.0064

Table 4. Relative Biological Effectiveness (RBE) of Neurtrons and γ-rays in Inducing Micronuclei (MN) in Lymphocytes

MN per cell	Neutron dose (Dn) required (cGy)*	γ-ray dose (Dr) required (cGy)*	RBE (Dr/Dn)
0.05	29.14$\pm$1.20	76.29$\pm$2.09	2.62$\pm$0.13
0.1	57.04$\pm$1.47	139.48$\pm$2.59	2.45$\pm$0.08
0.2	98.13$\pm$1.50	228.49$\pm$2.76	2.33$\pm$0.05
0.4	155.18$\pm$3.55	353.8 $\pm$2.95	2.28$\pm$0.06
0.8	242.44$\pm$0.55	530.72$\pm$3.08	2.19$\pm$0.14

*Calculated from best fitting linear-quadratic model.

systems are being developed for this purpose[14,15].

The characteristics of the dose-response relationship obtained with the CB method for human lymphocytes exposed in vitro to ionizing radiation have been evaluated by different researchers. The relationship of MN in CB cells with X-ray, gamma-ray or beta-ray dose is linear up to about 2 Gy, which is in contrast to the linear quadratic relationships usually observed for chromosome-type aberrations[7,8,10,16~18]. Prosser et al.[12] have demonstrated that up to 1 Gy of exposure the frequency of induction of MN per CB cell is very similar to that observed for the frequency of total aberrations, but beyond 1 Gy the level of aberrations becomes relatively higher. This could be explained by the fact that at low doses damaged cells will not contain

more than one acentric fragment, and this would have a finite probability of becoming a micronucleus. However, at higher doses, where a damaged cell may be expected to contain two or more acentric fragments, it becomes possible that more than one fragment will be incorporated in a micronucleus, especially if the fragments are very close each other. This will have the effect of depressing the extent of observable damage, and thus may explain why beyond 1 Gy the MN frequency dose-response remains vitually linear while the chromosome-type aberrations dose-response shows an obvious quadratic component.

The absolute MN frequencies quoted by Almassy et al.[5] are systemically lower than those shown by our γ-ray data. Differences in baseline frequencies among donors, in interpreting the critieria for scoring MN, in culture technique, and in fixing and staining procedures are possible explanations for differences in MN yields reported by our laboratories. It seems that a higher degree of international cooperation and standardization of the CB MN assay is required to eliminate the reported differences. Several recent technical developments may further enhance the use of the CB method for in vitro dosimetry. Identification of kinetochores within MN using anti-kinetochore antibodies[19] or centromeres using centromeric probes[20] provides a means of distinguishing MN containing whole chromosomes from MN containing acentric fragments, thus providing a better definition of the endpoint scored.

In conclusion, the ideal biological dosemeter should be rapid, easy and all the different radiation qualities should be covered by method[1]. From this viewpoint, the CB MN assay may have the potential to complement metaphase analysis of chromosomes for estimating chromosome damge in human lymphocytes following in vivo irradiation. Automation of the CB MN technique and dicentric chromosome analysis are real possibilities that would enhance the combined application of these methods for population monitoring.

ACKNOWLEDGEMENTS

REFERENCES

1. **Müller WU, Streffer C:** Biological indicators for radiation damage. Int J Radiat Biol 59:863-873, 1991

2. **Lloyd DC:** An overview of radiation dosimetry by conventional cytogenetic methods. In "biological dosemetry", ed, Eisert WG and Mendelsohn ML, pp 3-13, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, 1984

3. **Countryman PI, Heddle JA:** The production of micronuclei from chromosome aberration in irradiated cultures of human lymphocytes. Mutat Res 41:321-332, 1976

4. **IAEA, International Atomic Energy Agency:** Biological dosimetry: Chromosomal aberration analysis for dose assessment, Technical report 260, IAEA publications, Vienna, 1986

5. **Almássy Z, Krepinsky AB, Bianci A, Kotles GJ:** The present state and perspectives of micronucleus assay in radiation protection. A Review, Appl Radiat Isot 38:241-249, 1987

6. **Gantenberg HW, Wuttke K, Streffer C, Muller WU:** Micronuclei in human lymphocytes irradiated in vitro or in vivo. Radiat Res 128:276-281, 1991

7. **Kormos C, Koteles GJ:** Micronuclei in X-irradiated human lymphocytes. Mutat Res 199:31-35, 1988

8. **Ramalho A, Sunjevaric, I Natarajan AT:** Use of frequencies of micronuclei as quantitative indicators of X-ray-induced chromosome aberrations in human peripheral blood lymphocytes: comparison of two methods. Mutat Res 207:141-146, 1988

9. **Vral A, Thierens H, De Ridder L:** Study of dose-rate and split-dose effects on the in vitro microuncleus yield in human lymphocytes exposed to X-rays. Int J Radiat Biol 61:777-784, 1992

10. **Hall SC, Wells J:** Micronuclei in human lymphocytes as a biological dosemeter: Preliminary data following beta irradiation in vitro. J Radiol Prot 8:97-102, 1988

11. **Hubber R, Braselmann H, Bauchinger M:** Intra-and inter-individual variation of background and radiation-induced micronucleus frequencies in human lymphocytes. Int J Radiat Biol 61:655-661, 1992

12. **Prosser JS, Moquet JE, Lloyd DC, Edwards AA:** Radiation induction of microuclei in human lymphocytes. Mutat Res 199:37-45, 1988

13. **Thierens H, Vral A, De Ridder L:** Biological dosimetry using the micronucleus assay for lymphocytes: Interindividual differencies in dose response. Health Phys 61:623-630, 1991

14. **Fenech M, Jarvis LR, Morley AA:** Preliminary studies on scoring micronuclei by computerized image analysis. Mutat Res 203:33-38, 1988

15. **Tates AD, Van Welie MT, Ploem JS**: The present state of the automated micronucleus test for lymphocytes. Int J Radiat Biol 58:813-825, 1990

16. **Fenech M, Morley AA**: Cytokinesis-block micronucleus method in lymphocytes: Effect of *in vivo* ageing and low dose X-irradiation. Mutat Res 161: 193-198, 1986

17. **Fenech M, Morley AA**: Measurement of micronuclei in lymphocytes. Mutat Res 147:29-36, 1985

18. **Mitchell JC. Norman A**: The induction of micronu-clei in human lymphocytes by low doses of radiation. Int J Radiat Biol 52:527-535, 1987

19. **Fenech M, Morley AA**: Kinetochore detection in micronuclei: An alternative method for measuring chromosome loss. Mutagenesis 4:98-104, 1989

20. **Aleixandre C, Miller DA, Mitchell AR, Warburton DA, Gersen SL, Disteche C, Miller OJ**: p83H identifies sequences at every human centromere. Hum Genet 77:46-50, 1987

= 국문초록 =

방사선 조사량에 따른 인체 정상 림파구의 미세핵 발생빈도

원자력병원 치료방사선과*, 방사선의학연구실+

김성호+ · 조철구# · 김태환+ · 정인옹+
류 성 렬# · 고 경 환* · 윤 형 근*

원자력 시설 이용 증대에 따른 불의의 방사선 사고에 대비하여 방사선 작업종사자의 피폭시 진단을 위한 검사방법이 필요하다. 이 방법은 검사를 위한 검체의 채취가 용이하고, 짧은 시간내에 간편하게 많은 sample을 처리하여야 한다는 조건을 만족시켜야 한다. 인체의 다양한 조직 및 세포 중에서 위의 조건을 만족시킬 수 있는 말초 혈액의 림파구는 비교적 방사선에 대한 감수성이 높다고 알려져 있으며, 채집 또한 용이하여 생물학적 선량 측정의 도구로써 이용가치가 높아 방사선 작업 종자사나 피폭 가능성이 있는 사람의 screening test에 사용될수 있다. 정상인에 있어서의 림파구내 미세핵 존재 여부와 방사선 피폭량에 따른 미세핵 발생빈도를 시험관내 실험을 통하여 표준화시켜 향후 방사선 피폭시 피폭선량을 역으로 산출해 낼 수 있는 방사선 장해의 평가 기술 개발의 기초자료를 마련하기 위하여 본 실험을 시행하였다.

정상인으로 부터 혈액을 채취하여 림파구만을 Ficoll-Hypaque gradient 방법으로 추출하여 배양한 다음, 본 치료방사선과의 중성자 치료기(MC-50, scanditronix)와 Co-60 teletherapy unit (Theratron-780, AECL)를 이용하여 방사선 조사를 시행하였다. Cytokinesis-block method를 이용하여 첫번째 분열을 한 림파구에서 미세핵(Micronucleus)을 현미경을 통하여 계수한 다음, 이의 선량-반응 관계식을 linear-quadratic model을 사용하여 구하고, 이를 근거로 하여 gamma-ray에 대한 중성자의 Relative biological effectiveness(RBE)를 산출하였다.

방사선에 피폭되지 않은 림파구의 미세핵 발생빈도는 binucleated cell 한 개당 0.013 ± 0.0002 로써 사람에 따라 통계학적으로 큰 차이를 보이지 않았다. 그림 2와 3에서 보는 바와 같이 개개인으로 부터 얻은 data는 감마선과 중성자선 모두에서 선량-반응 곡선의 linear-quadratic equation에 잘 일치하였다. 감마선과 중성자선 모두에서 선량에 따른 미세핵의 발생빈도는 선량이 높을수록 비례하여 증가하였는데, 감마선의 경우에는 $r^2=1.000$, $x^2=0.7074$, $p=0.95$ 였으며, 중성자선인 경우에는 $r^2=0.996$, $x^2=7.6834$, $p=0.11$ 였다. 이를 linear-quadratic model로 분석하면, 가장 적합한 선은 감마선인 경우에는 $y=(0.31 \pm 0.049) D+(0.0022 \pm 0.0002) D^2+(13.19 \pm 1.854)$ 였으며, 중성자선인 경우에는 $y=(0.99 \pm 0.528) D+(0.0093 \pm 0.0047) D^2+(13.31 \pm 7.309)$ 였었다. 감마선에 대한 중성자선의 상대적 생물학적 효과비(RBE)는 $y=aD+bD^2+c$를 다음과 같은 식으로 변형시켜 계산하였다.

$$D=\frac{[-a \pm \sqrt{a^2-4b(c-y)}]}{2 \times b}$$

미세핵 발생빈도가 세포당 0.05와 0.8 사이에서의 중성자선의 상대적 생물학적 효과비는 2.37 ± 0.17 이었다.

이상의 결과를 종합하여 볼 때 선량에 따른 미세핵 발생빈도는 기존의 방사선 감수성 test의 결과와 대동소이하여, 앞으로 방사선 감수성을 측정하는 방법으로 이용할 수 있으며, 또한 실험방법이 비교적 간단하며 짧은 시간에 결과를 도출할 수 있어 생물학적 선량측정 도구로써 널리 이용될 수 있을 것으로 생각되어 진다.