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Malformation Effects in ddY Mice Irradiated at Two Stages in the Preimplantation Period

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ddYマウスの着床前期の二つの時期における放射線の催奇形性に関する研究

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従来から着床前期の胎児の放射線影響は、「all or none」であると提唱されてきた。しかし、著者らの先行研究により、着床前期でも時期によって奇形が生じ、しかも器官形成期よりも感受性が高いことが明らかにされた。従って、本研究では、マウスの種による特異的な影響かどうかを検討するため、妊娠ddYマウスを用いて受精後24時間と48時間にX線(CLINAC 6-100 Varian)線量0.1Gy, 0.5Gy, 0.75Gy, 1.5Gyおよび3Gy照射群(線量率: 20 cGy/min)に分けて研究を行った。これらの着床前期に放射線照射を行い、着床前死亡、胚死亡、胎児死亡、胎児体重、外表奇形等を受精18日目に解剖し、観察した。本研究により、胚死亡については、器官形成期に比べ着床前期の方が高感受性であることが明らかにされた。また、着床前死亡と胚死亡については、受精24時間後が最も感受性が高かった。受精24時間後は、受精卵の2細胞期であり、この時期の照射により、外脳症、口蓋裂、無眼球症などの様々な奇形が出現された。本研究により、受精24時間後におけるしきい線量は0.5Gyで、器官形成期における奇形のしきい線量は1.4Gyである。従って、奇形誘発についても器官形成期に比べ着床前期の方が高い感受性を示した。しかし、本研究では、受精48時間後の照射による奇形誘発は認められなかった。

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Key words: Radiation, Malformation, Embryonic death, Preimplantation, Pre-morula cells

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Introduction

There have been many experimental studies on the developmental effects of radiation in mouse embryos. Their major conclusions were that the mouse embryos at the preimplantation stage were highly sensitive to lethal irradiation effects, whereas there was very little increase in malformation frequency due to irradiation during the preimplantation period. Therefore, the conclusion reached by many publications is that the embryonic developmental effects on the embryo during preimplantation followed the "all or none" rule, that is, the embryos affected by radiation were dead or normal¹⁾⁻⁷⁾. However, Pampfer, Streffer and Muller, recently have reported steeply increasing frequencies of malformations in Heiligenberger strain mice which were irradiated at various stages during preimplantation⁸⁾⁻¹⁰⁾. Furthermore, Muller et al., describe that only in the case of both parents being of Heiligenberger origin was a pronounced increase in malformation frequency observed after irradiation of embryos in the 1-cell stage. However, No statistically significant increase in radiation-induced malformations was obtained in the F1 fetuses when the father was Heiligenberger and the mother C57BL¹¹⁾. They proposed that the possibility that radiation exposure during the preimplantation period in some mouse strains induced malformations should not be neglected. In the Heiligenberger mice, gastroschisis was frequently induced by irradiation during preimplantation stages. The frequencies of radiation-induced malformations varied both with the strain and with the stage of preimplantation. The preimplantation stage is a very important stage for radiological protection, because it is difficult to voluntarily avoid irradiation to embryos at this stage. Further studies are needed in order to resolve the problem of whether there is an increase in frequencies of malformations due to irradiation during preimplantation periods or not. We previously studied the relationship between preimplantation stage and malformation in ICR mice¹²⁾. Here we further study using the ddY mouse to examine external

malformations and other effects in (ddY mouse)embryos irradiated at two stages during preimplantation.

Radiation at the preimplantation stage is thought to cause a hereditary effect. Gastroschisis occurs frequently by X-rays during preimplantation period in Hillebrandts' report⁽¹³⁾. The ddY strain of mice have frequently been used in many experimental studies of malformations^{(14),(15)}. We investigated the effects on embryos at 24 hp.c. and 48 hp.c. (hours post-conception), which correspond with a two-cell stage embryo and a less than 8-cell stage precompacted embryo⁽¹⁶⁾. In many previous studies, relatively high doses of more than 0.5 Gy were used, leading to the conclusion that lethal effects were dominant on embryos/fetuses⁽¹⁾⁻⁽³⁾. Therefore, we irradiated the embryos during preimplantaion with relatively low doses.

Methods and Materials

1. EXPERIMENTAL ANIMALS AND MATING PROCEDURE

We used ddY mice housed at a temperature of 21-23°C and a relative humidity of 50 to 70% with a 12-hour light-dark cycle (lights on at 8:00 and off at 20:00).

The ddY mice have been fertilized individually since 1963 from the Japan National Institute of Health. Coatcolor is albino, and the generative descendant and the development of multiplication are good. And ddY mice are widely being used for the bioassay and other research in Japan.

A closed colony of ddY mice was purchased from SLC Japan, Inc.

The mice were given free access to food (CA-1, CLEA Japan Inc.) and to tap water. One or two female mice and one male mouse of the same age range were mated for exactly three hours from 8:00 to 11:00. The female mice in which vaginal plugs were detected were assumed to have become pregnant at 10:00^{(17),(18)}.

2. IRRADIATION WITH X-RAYS

The pregnant mice were placed in plastic cages for exposure, and were treated with a single whole-body X-radiation at 0.1 to 3 Gy with a dose rate of 20cGy/min. We used a 6 MeV X-radiation source (CLINAC 6-100 Varian). The time of exposure for embryos was 24 or 48 hpc. The total number of dams and live fetuses observed in this study were 278 and 2,432 for irradiation, respectively, while 48 control dams and 278 live fetuses served as unirradiated controls.

3. OBSERVATION OF EXTERNAL MALFORMATION AND OTHER EFFECTS

After irradiation, the pregnant mice were sacrificed by cervical dislocation on day 18 of gestation. The total num-

bers of corpus luteums in the ovaries and the total number of implantation consisting of and of live and dead embryos/fetuses were counted. The implantation was defined by a ratio of the number of implantation/the number of corpus luteum. The live fetuses were removed from the uterus and examined for external malformations under a dissecting microscope. The body weight and sex of each fetus were also determined.

4. STATISTICAL METHODS

In studying teratological effects, it is not appropriate to consider the fetus/embryo to be an experimental unit⁽¹⁹⁾. Instead, the litters (pregnant mice) were used as the experimental unit in the statistical analysis of the experimental data. In the per litter analysis, an average fetal response within a litter was calculated. For statistical tests, we used nonparametric methods, with Kruskal-Wallis tests for comparison among dose groups and Wilcoxon tests for comparisons between two groups⁽²⁰⁾. This is because the embryo/fetus binary response data do not show a normal distribution.

In addition, we used logistic regression analysis, which models the relationship between radiation dose and a binary response such as frequency of malformation or embryonic/fetal death. The linear logistic regression model has the form,

$$\text{logit}(p_{ij}) = \log(p_{ij}/(1-p_{ij})) = \alpha + \beta D,$$

where p_i is the probability that a binary response of the i -th event occurs, D is dose, and α and β are regression parameters. P_i is x_{ij}/n_{ij} in each litter, where n_{ij} is the number of live fetuses of the j -th litter in the i -th dose group and x_{ij} the number of the events. Fitting the binary logistic regression model was carried out using the SAS LOGISTIC procedure^{(21),(22)}. Based on the logistic regression analysis, the threshold dose of embryonic death or malformation was obtained as the dose giving a probability of 5% or two-thirds (2/3) of 10%^{(23),(24)}.

Results

1. INTRAUTERINE DEATH

Prenatal deaths of embryos/fetuses were divided into three categories: preimplantation death, post-implantation embryonic death and fetal death. The number of preimplantation deaths in each mouse was calculated by subtracting the number of dead and live embryos/fetuses from the number of corpus luteums in each pregnant mouse. In implantation sites, placental remnants and resorption of embryos were identified as post-implantation embryonic death. Maceration of fetuses was identified as fetal death. The implantation rate and the mortalities during embryonic and fetal periods in mice irradiated at various stages of preimplantation are shown in Tables 1

Table 1. Embryonic/fetal death and fetal body weight of ddY mice irradiated at 24 hpc in the preimplantation period.

Dose (Gy)	No. of Dams	No. of Implantations (%)	No. of Embryonic deaths (%)	No. of Fetal deaths (%)	No. of Live Fetuses (%)	Fetal body Weight (g)	
						Mean \pm SD male	Mean \pm SD female
Control	48	612 (99.2)	45 (7.4)	1 (0.16)	561 (92.4)	1.38 \pm 0.11	1.32 \pm 0.10
0.1Gy	20	384 (66.7) b	41 (15.6) a	0	216 (84.4) a	1.10 \pm 0.17c	1.10 \pm 0.10c
0.5Gy	25	499 (63.0) b	60 (19.0) a	0	255 (81.0) a	1.20 \pm 0.09	1.21 \pm 0.10
0.75Gy	25	534 (53.3) b	129 (45.6) b	0	155 (54.4) b	1.50 \pm 0.08	1.42 \pm 0.14
1.5Gy	24	424 (56.6) b	101 (41.7) b	0	140 (58.3) b	1.57 \pm 0.06	1.60 \pm 0.15
3Gy	22	408 (46.4) b	69 (37.8) b	0	123 (62.2) b	1.11 \pm 0.03c	1.12 \pm 0.13c

a: Significantly different from control group $p < 0.05$ by Wilcoxon nonparametric test.

b: Significantly different from control group $p < 0.01$ by Wilcoxon nonparametric test.

c: Significant at the 1% probability level by Student's t-test.

(): The number in the parentheses is a percentage. For example, the rate of implantation in the womb was calculated as dividing the fertilized embryonic egg (the count of the number of corpus luteum) by the number of preimplantation. An embryonic death rate was the number of death divided by the number of implantations in the womb. A fetal death rate was a ratio of the number of dead fetuses to the total number of embryonic and fetal death. A live fetus rate was a ratio of the number of live fetuses to the number of implantations.

We used Kruskal Wallis tests for preimplantation, embryonic and fetal death and there was a significant difference from the control group. We used the t-test for statistical analysis of the fetal body weight and it also showed a significant difference from the control group.

Table 2. Embryonic/fetal death and fetal body weight of ddY mice irradiated at 48 hpc in the preimplantation period.

Dose (Gy)	No. of Dams	No. of Implantations (%)	No. of Embryonic deaths (%)	No. of Fetal deaths (%)	No. of Live Fetuses (%)	Fetal body Weight (g)	
						Mean \pm SD male	Mean \pm SD female
Control	48	612 (99.2)	45 (7.41)	1 (0.16)	561 (92.4)	1.38 \pm 0.11	1.32 \pm 0.10
0.1Gy	21	387 (69.5) b	25 (9.52) a	0	234 (90.5) a	1.28 \pm 0.12	1.32 \pm 0.07
0.5Gy	20	408 (58.8) b	56 (23.33) b	0	184 (76.7) b	1.38 \pm 0.12	1.37 \pm 0.06
0.75Gy	20	368 (67.4) b	72 (29.03) b	0	176 (71.0) b	1.20 \pm 0.08	1.26 \pm 0.13
1.5Gy	25	515 (52.4) b	98 (36.70) b	2 (0.37)	170 (63.0) b	1.30 \pm 0.14	1.25 \pm 0.10
3Gy	28	604 (56.3) b	212 (62.4) b	0	128 (37.6) b	1.20 \pm 0.10c	1.25 \pm 0.06c

a: Significantly different from control group $p < 0.05$ by Wilcoxon nonparametric test.

b: Significantly different from control group $p < 0.01$ by Wilcoxon nonparametric test.

c: Significant at the 1% probability level by Student's t-test.

We used Kruskal Wallis tests for preimplantation, embryonic and fetal death. The results showed a significant difference from the control group. We used the t-test for statistical analysis of the fetal body weight. The results showed a significant difference from the control group.

and 2. The implantation rate of the non-irradiated control mice was 99.2 %. In the mice irradiated at 24 and 48 hp.c., the implantation rates significantly ($p < 0.01$) decreased at doses of 0.1Gy or greater.

The frequency of embryonic death in the control mice was 7.4%. The frequencies of embryonic death in mice irradiated at two stages of preimplantation increased significantly ($p < 0.01$). Particularly in mice irradiated at 24 and 48 hp.c., were strong dose-response relations for embryonic mortality found ($p < 0.01$). The frequencies of fetal death in irradiated mice at various stages of preimplantation were not significantly different from those in control mice. Regarding the mortalities during the preimplantation and embryonic periods, this study showed that the embryos irradiated at 24 hp.c. and irradiated at 48 hp.c. were similarly sensitive to radiation.

2. EXTERNAL MALFORMATIONS

The incidence of external malformations observed in fetuses irradiated at 24 hp.c. is shown in Table 3 and Fig 1. External malformations such as exencephaly, cleft palate, abdominal hernia, open eyelid, anophthalmia and abnormal tail, were observed in fetuses irradiated at 24 hpc. However, postaxial incomplete polydactyly of the forelimb was removed from the calculation of the malformation rate, since it might not be a malformation of the absentia types. Open eyelids were also observed in control fetuses; however, other external malformations were not observed in control fetuses. On the other hand, in fetuses irradiated at 48 hp.c., external malformations were not observed.

The dose parameter of the logistic regression model performed a statistical test that measured whether the total incidence of all types of malformation increased with dose. Us-

Table 3. Numbers of fetuses bearing external malformations in mice irradiated at 24 hpc in the preimplantation period.

Type of malformation	Control	0.1Gy	0.5Gy	0.75Gy	1.5Gy	3Gy
Exencephaly	0	0	2	1	3a	4b
Cleft palate	0	0	2	2	2	1
Anomalies of leg	0	0	1	0	0	0
Open eyelid	1	2	0	1	1	1
Anophthalmia	0	0	0	3a	3a	1
Abdominal hernia	0	1	0	1	0	0
Anomalies of tail	0	1	4b	0	2	1
Total number of malformations	1	4	9	8	11	8
Total number of dams	48	20	25	25	24	22
Incidence of malformations (%±SD)	(0.17±0.11)	(1.85±0.24)	(3.52±2.37)	(5.16±3.21)	(7.85±3.25)	(6.50±1.92)
Total number of live fetuses	561	216	255	155	140	123

a: Significantly different from control group $p < 0.05$ by Wilcoxon nonparametric test.

b: Significantly different from control group $p < 0.01$ by Wilcoxon nonparametric test.

We used Wilcoxon tests for the malformation rate between each treatment groups and they were significantly different from control group.

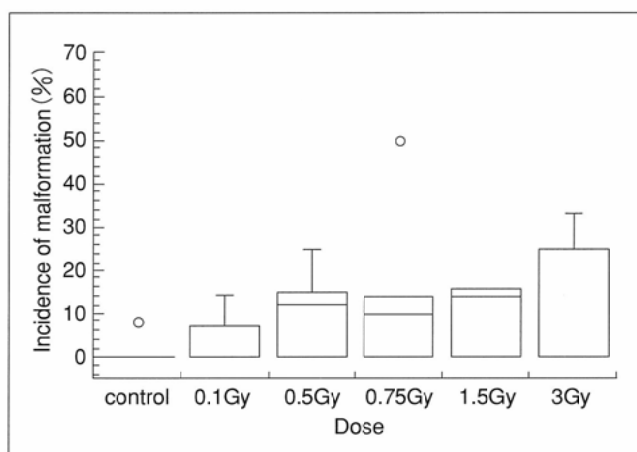


Fig. 1 The Incidence of malformation in ddY mice irradiated at 24hpc in the preimplantation period.

A significant ($p < 0.01$) dose-dependence was detected among all dose groups by the Kruskal Wallis test.

○: ○ shows the disconnection value which isn't in the box of litter effect.

ing the methods of Wilson JG²⁰⁾, the increase was significant (Wald χ^2 statistics with $p < 0.01$) for 24 hp.c. The litter size decreased with dose. For considering the litter effects, we added the deviation ($n_{ij} - \mu_i$) of each litter size n_{ij} from μ_i as an explanatory variable into the logistic regression [23], $\text{logit}(\pi_i) = \alpha + \beta D + \gamma(n_{ij} - \mu_i)$, where μ_i is the mean litter size among the i -th group.

The fitting results showed that the litter-size effect did not exert an influence on the dose effects. It was evident that malformations significantly increased with the dose at 24 hpc. However, there was no teratogenesis at 48 hpc.

3. FETAL BODY WEIGHT

The fetal body weights on day 18 of gestation are shown

in the seventh column in Tables 1 and 2. The body weights of female and male control fetuses were 1.323 g and 1.376 g, respectively. There was no significant difference in fetal body weights between irradiated and control mice, except for the body weights of mice irradiated with 0.1 and 3 Gy at 24 hp.c. and 48 hpc. Also, the sex ratios of the fetuses were not significantly affected by irradiation.

Discussion

It is difficult to estimate a threshold dose statistically because the problem of statistical type-two error always remains. In analyzing experimental data, it was useful to find an alternate index as a single value expressing a dose-response relationship. For analyzing a threshold dose-like dose response, we used the ED 5 and two-thirds of the ED10, which has been used to estimate threshold doses for deterministic effects^{22),23)}. These doses expressing the predicted values bases based on a logistic regression can be used for comparing each dose-response relationship in a quantitative way for a threshold-like dose response. The mice irradiated at 24 hp.c. had the lowest threshold dose of embryonic death, 0.075 Gy or 0.085 Gy. Rush et al. point out that threshold dose of embryonic death is 0.47Gy by the UNSCEAR report²³⁾. Ozhu and Makino point out that threshold dose of embryonic death is 0.42Gy. These results are similar to the results of this research²²⁾.

It has been reported that the preimplantation mouse embryo was most sensitive briefly after sperm entry into the oocyte²⁵⁾. Schlesinger et al. and Pampfer and Streffer reported that the one-cell-stage embryo showed sensitivity of preimplantation death and early postimplantation death in C57BL and Heiligenberger mice, respectively^{8),26)}. In in-vitro studies, the

embryo was very sensitive to radiation when the irradiation was carried out during the pronuclear stage^{27),28)}. In ddY mice, embryos at 24 hp.c. were at the two-cell stage and were located at the ampullar region of the oviduct. Fertilized eggs completed the first cleavage division with the 24 hours post conception²⁶⁾. In this study, the sensitivities for embryonic death decreased during the developmental stage during the preimplantation period. Similar changes in radiosensitivity have been reported in C57BL mice¹⁾ and Heiligenberger mice⁸⁾. The threshold dose of embryonic death in ICR mice irradiated at day 8 of gestation was 1.4 Gy in our previous study²⁸⁾. The relationship between incidences of all types of external malformations in mice irradiated at 24 hp.c. . The threshold dose of external malformations in the ddY mice irradiated at 24 hp.c. was 0.6 Gy, obtained on the assumption that it is the same as the 5% effective dose estimated by logistic regression. At the threshold dose of external malformations, only exencephaly and anophthalmia were observed. In ICR mice irradiated at day 8 of gestation, i.e., during organogenesis, the threshold dose for external malformations was 1.0 Gy as estimated in our previous study²⁸⁾. The studies carried out by Muller et al. reported that Heiligenberger mice had a significant elevated sensitivity of gastroschisis for irradiation during the preimplantation stage¹⁰⁾. Recently, Nagao has reported that the embryos during the preimplantation period were susceptible to ethylnitrosourea, which is a chemical mutagen²⁹⁾. The fertilized egg at 24hp.c. is the juncture when the first cleavage occurs²⁶⁾. It becomes for any organs to the embryonic cell of this stage is totipotence germ cell, too. Therefore, as for the irradiated fertilized egg at 24hp.c., it is thought that the various malformation can occur¹²⁾. Also, Pampfer and Streffer reported that irradiation of zygotes with neutrons or X-ray increased significantly the number of externally malformed fetuses, with a linear quadratic dose effects relationship and that the spectrum of observed malformations was larger after irradiation than in controls⁸⁾. In Juchaus¹³⁾' report, an attempt has been made to summarize our current understanding of the mechanisms whereby certain chemicals cause birth defects¹³⁾. It is clear that his current understanding of mechanisms whereby these agents cause teratogenic effects (birth defects) can vary dramatically from one agent to the next. Extremes include the folic acid antagonists, which are now well established as agents that produce birth defects

by virtue of potent inhibition of dihydrofolate reductase as a primary biochemical mechanism³¹⁾. Parental exposure to X-rays induced significant yields of dominant lethals, anomalies and tumors in the offspring. However, the incidence of death and anomalies were much lower than those induced by treatment of embryos during organogenesis with equivalent doses of X-rays³²⁾. In Hillebrandts' report¹³⁾, gastroschisis occurs with a high frequency in the mouse-inbred strain HLG compared with C57BL/6J mice. A suggestive linkage for a locus responsible for radiation-induced gastroschisis was found in a region of mouse Chromosome 7¹³⁾. In this study, no malformations were observed in the mice irradiated at 48 hp.c., at which stage the embryos were pre-morula at about the 6-cell stage. In the pre-morula embryos, damage cells could be replaced by the trophectoderm.

The inner cell mass either receiving no damage or fully repaired damages would have totipotency. After the 8-cell stage, compaction occurs among the cells and then each cell begins to have a specific function and morphology; therefore, many types of external malformations could occur. In the one-cell stage embryos, external malformations could be caused by genetic changes in the fertilized egg due to teratogens such as radiation^{9),25)}.

A difference between the mouse strains in the malformation induced to preimplantation stage is not detected for ddY and ICR mice¹²⁾. The malformation induction of the Heiligenberger mice (HLG/Zte) is high against 1Gy³³⁾. This result is the same as this research. However, Muller et al. reported that this was a strain specific phenomenon¹¹⁾. Therefore, Muller et al. refer to those experiments using the entirely different strain C57BL. As reported in their paper, they did not indeed find an increased malformation frequency after exposure of the zygote under exactly the same conditions as in the Heiligenberger experiments¹¹⁾. It might be that malformation occurred significantly with ddY mice and ICR mice. Future research to explore the mechanism underlying strain difference should be done in future.

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