

Non-linear Responses of *Hordeum vulgare* Germs to Low Doses of Ionizing Radiation

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Abstract - The induction of chromosome aberrations in *Hordeum vulgare* germs after irradiation is studied for the dose range of 10 to 1,000 mGy. The relationship between the frequency of aberrant cells and the absorbed dose is shown to be non-linear and has a dose-independent plateau within the range of 56-467 mGy where the level of cytogenetic damage is statistically significantly distinguished from the spontaneous level. The comparison of the goodness of the experimental data fitting with mathematical models of different complexities, using the most common quantitative criteria, demonstrates the benefit of the piecewise linear model over the linear and polynomial ones in approximating the cytogenetical disturbance frequency. The results of our study support the conclusion about indirect mechanism of chromosome aberrations induced by low doses or dose rates mutagenesis.

Key words : low doses, ionizing radiation, non-linear dose-effect, cytogenetic disturbances

INTRODUCTION

An estimation of biological effects produced by low-level radiation is a complex problem that includes many unsolved questions of modern biology. A correct estimation of the effects of low doses is one of the important topics of radiation biology since a new developing concept of radiation protection for humans and the biota should be based on a clear comprehension of how the consequences of low level exposure appear.

The results of the dose-response studies form the background for developing theories of induced mutagenesis. Therefore, the goals of the present work were (i) to investigate the induction of chromosome aberrations with doses ranging from 0 to 1 Gy in *Hordeum vulgare*

germs; and (ii) to examine the overall shape of the dose-effect curves and to test whether or not the cytogenetic disturbances yield linearly depends on a dose in this range.

MATERIALS AND METHODS

1. Test organism

Spring barley (*Hordeum vulgare* L., variety Zazerskiy 85), one of the most genetically studied crops (Constantin and Nilan 1982), was used. Barley is a convenient object for studies of induced chromosome aberrations because of its few ($2n = 14$) relatively large (6-8 μm) chromosomes which are easy to identify. A frequency of anaphase cells with chromosomal aberrations in the root meristem cells was used as the endpoint of the genotoxicity.

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2. Irradiation and root tip fixation

The seeds were soaked for 24 h in distilled water at 0 °C in the darkness to synchronize the cells' division. They were allowed to germinate for 12–16 h at 24 °C. γ -Rays were produced by a ^{137}Cs external source, which delivered γ -rays at a dose rate of 0.5 Gy h⁻¹. A dose range comprised between 0 and 1 Gy, namely: control, 10, 50, 100, 150, 200, 300, 500, 750 and 1000 mGy were applied in order to observe the overall shape of the dose-effect relationships on the chromosomal aberrations induction. After irradiation, 20–70 seedlings per dose point displaying a root length of ≈ 10 mm long were immediately fixed in acetoalcohol (1:3).

3. Cytogenetic analysis

Cytogenetic analysis at ana- and telophases was performed in temporal squashed preparations stained with aceto-orcein as described earlier (Constantin and Nilan 1982; Geras'kin *et al.* 2003). All slides were coded and examined blindly. In each slide, all ana-telophase cells (700–2700 cells per dose point) were scored to determine the fraction of cells with aberrations.

4. Statistical analysis

A volume of cytogenetic data picked up in the experiment was estimated and optimised by a method of empirical distributions analysis that allows us to find the optimum sample size required for an estimation of the examined parameters with a relative probable error at the given confidence level for each treatment variant (Geras'kin *et al.* 1994). A quality of data approximation with two different mathematical models was estimated by the Hayek criterion (Gofman 1990):

$$H = \sqrt{\frac{\mu(R_2^2 - R_1^2)}{1 - R_2^2}}, \quad R_2^2 > R_1^2$$

where R_1^2 and R_2^2 are multiple correlation coefficients for models 1 and 2, μ -degrees of freedom in model 2. H-statistics follows the Student distribution. After computing the H-value and choosing the statistical significance level, the conclusion can be drawn whether model 2 fits the experimental data significantly better than model 1. A choice of the model with an optimum ratio of

the complexity and goodness of data fitting was carried out by the criteria of structural identification T (Geras'kin and Sarapul'tzev 1993).

RESULTS

The results of the cytogenetic analysis are presented in Table 1. The aberrant cells frequency hardly changes in the dose range of 50–300 mGy and considerably exceeds the control level. However, the statistical analysis of the data with the Student test shows that the cytogenetic disturbance yields differ from the control confidently only when the radiation dose is equal or over 300 mGy. Meanwhile, the nonparametric Kolmogoroff-Smirnoff test, with the mean value of the aberrant cells frequency significantly ($p < 0.05$) exceeds the control level after irradiation at doses of 50 mGy and over.

In light of the conflicting results obtained from the common statistical procedures, other statistical methods especially meant for deriving a reliable statistical assessment of a small sample volume are worth consideration. One such tool is a bootstrap method applied in some of our previous works, in particular, for the analysis (Geras'kin *et al.* 1993) of the genetic consequences of the Chernobyl NPP accident. An idea for this method development was conditioned by an increasing recognition of the fact that the classical statistical procedures based on the method of maximum likelihood do not allow to derive all the available information from a sampling. In contrast to the traditional statistical methods, the bootstrap is based on a multiple handling of different parts of a sampling and the comparison of the information obtained in that way (Efron 1979). The bootstrap-estimates have such properties as an asymptotic optimality and local asymptotical minimaxity.

For every dose, a bootstrap-distribution of the mean values of the aberrant cell frequency was reconstructed by the Monte Carlo means. Averages and standard errors of these bootstrap-distributions are presented in Table 1 (the number of bootstrap-realizations $B = 100$). An application of a set of parametric and nonparametric tests to analyse the bootstrap-samplings shows that they are robust to a sufficient degree, i.e. their basic

Table 1. The aberrant cells frequency in irradiated barley germs calculated by common and bootstrap procedures

Dose, mGy	Cells scored	Aberrant cells frequency, (% ± st.er.)			
		Common method	Bootstrap method	CI	
0	1676	0.57 ± 0.25	0.592 ± 0.003	0.542 ÷ 0.642	Part 1
10	1396	0.74 ± 0.26	0.729 ± 0.003	0.675 ÷ 0.783	
50	2632	1.41 ± 0.25	1.427 ± 0.003	1.378 ÷ 1.476	Part 2 (plateau)
100	1898	1.49 ± 0.29	1.495 ± 0.003	1.441 ÷ 1.549	
150	1604	1.58 ± 0.27	1.590 ± 0.003	1.537 ÷ 1.643	
200	1295	1.42 ± 0.26	1.423 ± 0.003	1.370 ÷ 1.477	
300	1989	1.44 ± 0.22*	1.422 ± 0.002	1.374 ÷ 1.470	
500	665	1.76 ± 0.47	1.755 ± 0.005	1.658 ÷ 1.853	Part 3
750	674	2.97 ± 0.66*	3.015 ± 0.006	2.899 ÷ 3.131	
1000	1325	4.53 ± 0.56*	4.617 ± 0.005	4.511 ÷ 4.723	

*-Difference from the control is significant, Student test; $p < 5\%$
CI-95% confident interval for bootstrap-estimates

properties (the distribution law and values of the key parameters) do not change from one random Monte-Carlo realization to another and do not depend on the number of realizations (B). The mean values of the aberrant cell frequency obtained by the usual method lay within the limits of the bootstrap confidence intervals (Table 1).

The new estimates of variances reveal differences between samplings, which the classical statistical methods failed to detect. The dose range itself divides into three parts (Table 1) such that there are essentially different regularities of the cytogenetic disturbance yields within these dose ranges. The dependency of the response on the dose value is statistically significant within the range of 500–1000 mGy (Part 3), while this is not the case in the range of 50–300 mGy (Part 2). The absolute term of the linear regression model for Part 2 is significantly higher than the spontaneous level of the aberrant cell frequency; this actually confirms the presence of a plateau distinct from the control. Let's note, that an arising inference about the non-linear character of the dose-effect dependence follows from the experimental data only and not from any hypotheses, extrapolation models or other speculations.

The data on the aberrant cells frequency in Table 1 suggests an essential deviation of the dose dependency from linearity in the whole dose range and no dose dependency in the range of 50–300 mGy. Whether or not there is an important deviation from linearity that

actually exists in our experimental conditions? A correct answer to this question should be grounded on the comparison of goodness-of-fit of the experimental data for the cytogenetic disturbances occurrence by mathematical models of different complexities. The most interesting models in the case under consideration are linear and piecewise linear models. The latter model supposes the non-linearity of a dose dependency and the presence of a dose-independent plateau in a certain dose range:

$$F(D) = \begin{cases} a_1 + (a_2 - a_1)/D_1 * D, & D < D_1; \\ a_2, & D_1 < D < D_2; \\ a_3 + (a_2 - a_3)/D_2 * D, & D > D_2, \end{cases} \quad (1)$$

where $F(D)$ -aberrant cells frequency, (%); D_1 and D_2 -the lower and the upper limits of the plateau; a_1 , a_2 , a_3 -parameters.

The parameters of the linear model in this study were determined by the linear regression tools, while the piecewise linear model parameters were defined from the minimization of the following functional

$$U[a_1, a_2, a_3, D_1, D_2] = \sum_{i=1}^N (F_i(D) - F(D))^2, \quad (2)$$

which is the sum of the squared differences between the experimental data $F_i(D)$, $i \in \overline{1, N}$ and a family of specified parametric piecewise linear functions $\{F(D)\}$ by the method of a coordinatewise descent (Vasiliev 1988). Numerical experiments showed that the optimisation problem

$$U(a_1, a_2, a_3, D_1, D_2) \rightarrow \min$$

has one stable solution in a 5-dimensional functional space, and it is independent from an initial approximation. From the calculations, the dose-independent plateau lies within the $D_1 = 56$ mGy and $D_2 = 467$ mGy limits, the levels of the cytogenetic disturbances at a zero dose (spontaneous) and within the plateau are $a_1 = 0.56\%$ and $a_2 = 1.52\%$, accordingly.

It is common knowledge (Vapnik 1984) that a predictive reliability of a model falls down if the number of free parameters increases. This is why, a linear model is advantageous as one of the simplest models. As a result of this, the current practice of plotting data on a linear dose scale for a wide range of doses will mask any

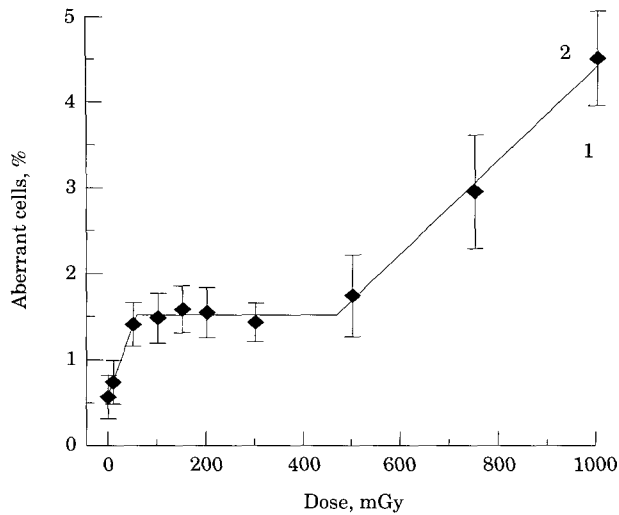


Fig. 1. Frequency of aberrant cells in barley germs exposed to low radiation doses and approximation of the data with linear (1) and piecewise linear (2) models.

effects occurring at low doses, which deviate from a linear or linear-quadratic dose-effect relationship. This technique may obscure the underlying relationships. Indeed, the dose-effect curve observed in our study at sufficiently low doses would not be detected if plotted together with high-dose data in a linear dose representation. In our opinion, the main advantage of the linear non-threshold (LNT) conception consists in these, purely mathematical properties of a linear function providing the current prevalence of the LNT, while its biological fundamentals are quite contradictory (Geras'kin 1995a; Tubiana 1998; Geras'kin and Sevankaev 2000; Pollycove and Feinendegen 2003).

It is apparent from the results presented in Fig. 1, even without using precise quantitative criteria, that the piecewise linear model (line 2 in Fig. 1) fits the data much better than the linear one. It is important, that the improvement of the quality of approximation is not reached on the account of the model complicating (the calculation of the structural identification criterion T (Geras'kin and Sarapul'tzev 1993) (see Table 2), which explicitly involves a penalty for model complexity, evidences that) but because it is possible to achieve a mutual conformity (or, in other words, functional isomorphism) between a biological phenomenon and its mathematical model with a set of the piecewise linear func-

Table 2. Comparison of approximation qualities of experimental data by various models

Model	SSR	F	R ² , %	T	H
Linear	1.35	62.7	88.7	0.34	14.3
Piecewise linear	0.03	1829.3	99.7	0.03	
Polynomial of degree 2	0.87	88.5	92.7	0.37	11.4
Polynomial of degree 3	0.49	139.2	95.9	0.33	8.5
Polynomial of degree 4	0.14	435.8	98.9	0.14	4.0
Polynomial of degree 5	4.25	7.2	64.3	6.37	25.6

SSR-squared sum of residuals; F-Fisher statistics

tions. The comparison of approximation quality that can be achieved by models of different complexity by the most common quantitative criteria (Table 2) testifies to it as well. From the Hayek criteria (Table 2) that permits an objective compare the goodnesses of experimental data fitting by different models, the piecewise linear model assuming nonlinearity of the dose-effect dependency and the presence of the plateau fits the cytogenetic disturbances occurrence in the low dose range much better than any other among the tested models (and, in particular, the linear).

DISCUSSION

The use of the LNT extrapolation in the radiological protection practice has been justified until now (Upton 2003) by a lack of reliable data on the effects of low doses, which is partly due to the difficulties of epidemiological studies and dosimetry problems with regards to low doses and dose rates. This approach, reducing the complex to the simple, has been favoured by the decision-makers, who are subject to the requirements of establishing an easily applicable regulatory framework. However, the presence of inducible defence systems suggests (Geras'kin 1995b; Tubiana 1998; Marples and Joiner 2000; Pollycove and Feinendegen 2003) that there should not be any simple linkage between exposure to a mutagenic agent and the incidence of mutations. A validity of the LNT concept needs to be verified since its experimental and epidemiological background is highly controversial (Geras'kin 1995a; Tubiana 1998; Geras'kin and Sevankaev 2000; Pollycove and Feinendegen 2003). The risk of this reductionism causes a great uncertainty concerning the capacity of the protection

system to effectively meet its objectives. Our findings give further evidence that the models founded on the LNT concept are inconsistent with the available experimental data as well. Consequently, it is necessary either to originate the consistent scientific base of LNT, or, having proved its scientific incompetence, to develop a new formalistic method for cost/benefit analysis at different levels of radiation exposure. From the practical point of view, the confirmation or the rejection of the LNT model will permit the construction of reliable models that will avoid the uncertainty concurrent for the modern scientific base of radiation protection norms for humans and biota.

It has also become apparent that the existing paradigm governing radiobiology cannot suggest a satisfactory explanation for the phenomena of radiation-induced genomic instability (Baverstock 2000; Morgan 2003) and the bystander effect (Seymour and Mothersill 2000; Morgan 2003). These effects cannot be explained in terms of structural or sequence changes to specific genes caused by the damage inflicted directly to the DNA by ionization events. But the experimental evidence to support the existence of both effects is unassailable now. This makes epidemiological studies particularly difficult to interpret since the relevant aspect of the genetic background is uncharacterized. Future studies will provide a greater clarification of the complex interrelationships that are involved.

For human lymphocytes, an increase of the aberration frequencies above the control values commences at low LET radiation doses of about 30–50 mGy (Pohl-Ruling *et al.* 1983; Lloyd *et al.* 1988; Lloyd *et al.* 1992). In the present study, a steep increase of the irradiation effect is observed for the same (50 mGy) dose. For higher doses we found the plateau (50–300 mGy). The deviation from linearity and drastic slope modification of the dose–response relationship might be due to an efficient threshold repairing system (Geras'kin 1995b), probably triggered by a certain dose below 50 mGy. By the inclusion of inducible radioprotective mechanisms in the radiobiological models it was possible to explain the plateau in dose–response relationships for cytogenetic disturbances (Oudalova *et al.* 2002) and neoplastic transformations (Schollnberger *et al.* 2001). It is therefore not surprising that the dose–response relationships for

cytogenetic disturbances can significantly deviate from the linear. So, the dose–effect curve for the range of 0–1000 mGy definitely can't be considered linear. This curve type is similar to the dose–effect curves described elsewhere (Luchnik and Sevankaev 1976; Takahashi *et al.* 1982; Zaichkina *et al.* 1992; Marples *et al.* 1997; Shmakova *et al.* 2002; Zaka *et al.* 2002) in animals and plants, characterized by a pronounced effect (hypersensitive response) in the range of low doses. Therefore, such a shape of the dose–response relationship is evidently not accidental and reflects qualitative singularities of a cell's response within the low dose range.

Up to now, an overwhelming point of view has assumed (ICRP 1990; Chadwick *et al.* 2003) that the direct induction of alterations in the genetic structure is paramount for the deleterious biological effects of radiation in a wide dose range, including low doses. However, plenty of experimental studies emphasize that a regulation of gene expression plays a very important role in forming an adequate cell response to low dose exposure (Geras'kin 1995b; Azzam *et al.* 1998; Schofield 1998; Baverstock 2000). As a genes expression can be changed (Azzam *et al.* 1998) by signals from neighbouring cells, perturbations of the gene expression pattern do not necessarily have to be caused by damage to the DNA. The collective cell's involvement in the response to a low dose exposure unambiguously rejects the possibility of interpreting these findings from the positions of somatic mutagenesis and determines the natural thresholds of the observed effects. The pronounced nonlinearity of the cell's response to low doses testifies that the genetic effects within this range are related to the peculiar features of the realisation of cellular response to a weak exposure rather than the direct damaging effect of ionizing radiation or other factors of a physical or chemical nature. The previous results of other authors (Dubrova *et al.* 1996; Marples *et al.* 1997; Schofield 1998; Nagasawa and Little 1999; Marples and Joiner 2000; Baverstock 2000; Seymour and Mothersill 2000; Shmakova *et al.* 2002; Kovalchuk *et al.* 2003) obtained from different objects and tests–systems support this statement.

An adaptive response to a low dose exposure is a fundamental biological phenomenon shown in many objects belonging to different taxonomic kingdoms–bacteria,

plants, insects, fish and mammals, –both *in vitro* and *in vivo* (Geras'kin 1995b; Koterov and Nikol'sky 1999; Sasaki *et al.* 2002). If toxic agents irrespective of their origin cause DNA damage, at low concentrations they should principally be considered also as stimulating the physiological DNA damage control system. The lowest dose or dose rate value, which is still capable of inducing damage control system, can be considered as a threshold of significant damage for a cell. Since the non-radiation DNA damage far outweighs (Sarapul'tzev and Geras'kin 1993; Lucas *et al.* 1999; Pollycove and Feinendegen 2003) damage from a low dose and low dose rate of low-LET radiation (for example, the ratio of metabolic DNA damage to radiation DNA damage from a low-LET background of 10 mGy y⁻¹ is 10⁶ (Pollycove and Feinendegen 2003)), the low-dose induction of the DNA damage control system affects mainly the DNA damage from non-radiation sources. So, the dose-effect curve in this range definitely can't be considered linear.

Since the regularities of radiation-induced structural mutations are a cytogenetic appearance of the fundamental principles of the organization and functioning of the eukaryotic genome, the experimentally observed (Marples *et al.* 1997; Nagasawa and Little 1999; Seymour and Mothersill 2000; Shmakova *et al.* 2002) hypersensitive response to low doses of a number of physical and chemical factors contrasts with the high reliability of genome intrinsic for the eukaryotic organisms (Sarapul'tzev and Geras'kin 1993). Such a sensitivity to weak disturbances of environmental parameters is of adaptive importance and directed (Geras'kin 1995b) towards an increase of the probability of cell population survival by means of: (i) activation of the processes reducing the amount of genetic damages (adaptive response); (ii) programmed death of "unwanted" cells, that are no longer needed by the organism or bearing potentially dangerous alterations (apoptosis); (iii) increase of the genetic diversity in the cells (SOS-repair, coherent transposition of mobile genetic elements, genomic instability) with a subsequent selection of the most suited variants.

A non-specificity of the regularities studied and a wide spectrum of the objects for which these are observed provide evidence that we are dealing with a general biological phenomenon. The basis for the cellular res-

ponse to low-dose irradiation and a weak exposure of other external factors is fundamental evolutionary-conserved mechanisms for ensuring stability of living systems that were developed long before multicellular organisms emerged. In fact, ionizing radiation has become a widely used tool for studying cellular and organism responses for low-level external impacts, and it serves to enhance our understanding of the adaptive process as well. Just as the phenomenon of DNA molecule repair discovered by radiobiologists has gone far beyond the scope of this science and is undoubtedly for general biological importance, the established radiobiological studies regularities of the biological effect of low-dose ionizing radiation are not artefact or some exotic "anomalous" reaction but one of the natural manifestations of the fundamental (being a basis for life) mechanisms for ensuring the living systems resistance and the possibility of their adaptation to the varying conditions of the environment.

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