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Research Article

Chromosome aberrations, micronuclei, and activity of superoxide dismutases in human lymphocytes after irradiation in vitro

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Abstract. The goal of this study was to provide data on the dose-dependent production of dicentrics and micronuclei in human lymphocytes irradiated with 22.6 MeV protons and to estimate the possible contribution of intracellular superoxide dismutases (SOD) to the relative biological effectiveness (RBE) of protons. For the dose-response study, heparinized whole blood of a healthy volunteer was irradiated with protons and X-rays employing radiation doses of 0.5–4 Gy. Three biological endpoints were analyzed: chromosomal aberrations, micronuclei, and specific activity of cytosolic (CuZnSOD) and mitochondrial (MnSOD) superoxide dismutases in harvested human blood cells. Dicentric dose-response curves fit a linear-quadratic form ($\alpha = 0.094 \pm 0.006$, $\beta = 0.032 \pm 0.001$) induced with X-rays and ($\alpha = 0.119 \pm 0.057$, $\beta = 0.029 \pm 0.014$) for 22.6 MeV protons. Protons were more effective than X-rays in producing exchanges, particularly at 0.5 and 1 Gy. In contrast to X-ray irradiated samples where a significant increase in the specific activity of MnSOD was recorded (up to a radiation dose of 1 Gy), irradiation with protons markedly reduced its activity. As a consequence of the reduced activity of MnSOD, the chromosomal dose-response curve became quadratic. The RBE for dicentrics varies with dose (from 2.2 to 1.01) and reduced activity of MnSOD is an important contributor to the RBE of protons. SODs, particularly MnSOD, play an important role in defending DNA from reactive oxygen species. A reduced activity of SOD, particularly MnSOD, is an important contributor to the RBE of protons.

Key words. Human lymphocytes; chromosome aberrations; micronuclei; superoxide dismutase; X-ray; proton.

Introduction

Ionizing radiations produce a range of lesions in DNA including base and sugar damage, single- and double-strand breaks, DNA-DNA, and DNA-protein cross-links. Of these lesions, DNA double-strand breaks (dsbs) are considered especially important, with evidence suggesting that both their initial or unrepaired level may be related to cell mortality. Single-strand breaks are of little importance for cell survival, although base damage and single-strand breaks could produce potentially lethal lesions [1].

Ward and coworkers [2–6] have suggested mechanisms whereby complex damage to intracellular DNA is

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caused by multiple radical attack on local sites. Ionizing radiation damages both single and multiple sites in DNA, with the range of radical migration limited by the presence of free radical scavengers. Currently, the most developed biological indicator of exposure to ionizing radiation are chromosomal aberrations (CAs) in peripheral blood lymphocytes [7–9]. Biological dosimetry is performed by measuring chromosome exchanges in mitotic lymphocytes which are produced in a dose-dependent manner [10–13]. Estimation of chromosome damage can be obtained by scoring micronuclei (MN) derived mostly from acentrics excluded from daughter nuclei at ana-telophase. The method is easier and faster than scoring of dicentric chromosomes and has been suggested as an alternative method for determining radiation exposure [14–19]. Since some of the damage induced by ionizing radiation results from the high-level action of free radicals, the activity of superoxide dismutases (SODs) should have an important role in defending DNA from reactive free radicals. SODs catalyze dismutation of superoxide radicals $O_2^{\cdot -}$ into H_2O_2 plus O_2, thus participating, with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In mammalian cells, the process of dismutation is catalyzed by two distinct enzymes, mitochondrial (MnSOD) and cytosolic (CuZnSOD) superoxide dismutase [20]. A deficiency in antioxidant enzymes and failure to repair radiation-induced damage may be associated with increased radiosensitivity [6, 21, 22].

The aim of this investigation was to provide data on the dose-dependent production of dicentrics and MN in human lymphocytes irradiated with protons and to estimate the indirect role of intracellular SODs on the yield of radiation-induced DNA damage.

Material and methods

Irradiation. The proton irradiation was performed with the 15 MV Tandem Accelerator in the INFN-Laboratori Nazionali del Sud (Catania). The entire experiment was performed with a 3.5-cm-diameter round beam. Dose homogeneity was obtained by scattering the protons through a 250-μm-thick lead foil and 150-μm-thick brass foil located 155 and 210 cm from the distal part of the collimator, respectively.

For the dose-responses, heparinized whole blood samples were irradiated in plastic chambers (outer dimensions $28 \times 28 \times 5$ mm) in a central portion of an unmodulated beam at the beginning of the peak. The dose-response was evaluated using quantification of chromosomal aberrations, MN, and activity of SODs, employing doses from 0.5 to 4 Gy at a dose rate of 1 Gy/min. Within the whole experiment, the dose rate varied about 4%, but each dose was delivered at a constant dose rate. Angular scattering of protons and energy loss straggling were neglected. The samples were irradiated at room temperature.

For comparisons, aliquots of 1.5 ml whole blood of the same donor were put into a sterile plastic test tube placed in a plexiglas container 15×15 cm and irradiated using 300 kVp X-rays, 10 mA, 2.7 mm Cu HVT. The radiation doses employed were 0.5–4 Gy, the dose rate was 1 Gy/min, the dimensions of the radiation field were 20×20 cm, and the distance from the source 74 cm. Samples were irradiated at room temperature.

Blood culture, chromosome, and MN analysis. Six hours after irradiation, three lymphocyte cultures were established for each sample irradiated with protons, as well as three lymphocyte cultures from each dose of X-irradiated sample. Cultures containing 0.5 ml whole blood, 6 μM BdUR, 8 ml of RPMI-1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, and 2.4 μg/ml phytohemagglutinin were kept for 48 h at 37 °C. Fixation of the cultures and preparation of the slides were carried out according to the FPG method [23]. After staining, about 200 well-spread and complete first-division metaphases per culture were analyzed for unstable chromosome-type aberrations, i.e. dicentric and polycentric chromosomes, centric rings, and excess acentrics. The scoring criteria were based on the IAEA recommendations [9]. Chromosomal aberrations were scored on FPG stained slides using a Zeiss microscope at magnification 16×100 or $16 \times 100 \times 1.25$ when necessary. The slides were coded with identification numbers and were scored blind.

For MN preparation, the cytokinesis block method of Fenech and Morley [24, 25] was followed with some modifications. Cytochalasin B at a final concentration of 6 μg/ml was added to the samples after 48 h of culture and the lymphocyte cultures were incubated for a further 24 h more. Seventy-two hours after culture initiation, cells were spun down, the medium was removed, and the cells were washed with PBS and fixed in methanol:acetic acid (3:1) after 5 min of mild hypotonic treatment (0.56% KCl + 0.9% NaCl mixed in equal volumes). Slides were air-dried and stained in alkaline Giemsa (2%). At least 1000 BN cells per sample were scored. All slides were analyzed with a Zeiss microscope ($\times 400$ or $\times 1000$ when necessary). In the MN study, a minimum of 1000 lymphoblasts were scored to evaluate the percentage of cells with one, two, three, four or more than four nuclei. A cytokinesis block proliferation index (CBPI) was calculated according to Surralles et al. [26] as follows: $CBPI = MI + 2MII + 3(MIII + MIV)/N$, where MI–MIV represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

Enzyme assays. To find a relationship between modulation of SOD activity and CA, as well as MN, aliquots of irradiated whole-blood samples, for each dose, were

Table 1. Dose-response (22.6 MeV protons, dose rate 1Gy/min).

Chromosomes

Dose (Gy)	Number of cells scored	Dicentrics per cell (y)	Exchanges per cell	Excess acentrics per cell	σ^2/y	U	Distributions of dicentrics between cells (number of cells with indicated number of dicentrics)							
							0	1	2	3	4	5	6	7	8
0	2894	0.001	0.001	0.001	–	–	2891	3							
0.5	434	0.106	0.14	0.1	1.18	−0.507	393	37	3	1					
1	329	0.284	0.32	0.09	1.33	−0.503	273	48	6	1	–	1			
2	290	0.365	0.47	0.09	1.65	−0.501	222	44	12	10	2				
3	188	0.62	0.98	0.24	2.58	−0.497	138	16	18	8	4	2	–	2	
4	282	1.04	1.62	0.62	3.27	−0.495	188	22	26	16	10	4	4	10	1

Micronuclei

Dose (Gy)	Number of BN cells scored	Number of micronuclei	MN/1000 BN cells	Micronuclei distribution						Proliferation index (CBPI)
				0	1	2	3	4	5	6	
Control	1098	24	22	1076	21	1					1.99
0.5	1868	162	87	1726	126	12	3	1			1.88
1	1794	310	173	1555	188	34	14	3			1.79
2	1224	352	288	990	152	54	22	3	3		1.84
3	1230	728	592	850	180	100	64	26	8	2	1.66
4	876	760	868	549	106	95	65	39	19	3	1.66

SODs

Dose (Gy)	CuZnSOD (U/mg protein)	MnSOD (U/mg protein)
Control	147.2	19.5
0.5	100.4	16.1
1	72.9	14.8
2	68.3	13.7
3	54.4	13.2
4	45.9	11

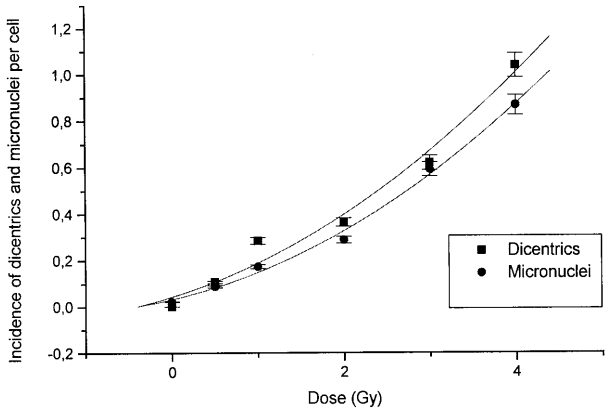

Figure 1. Dose dependence of dicentrics and micronuclei obtained by 22.6 MeV protons. Symbols represent incidence of dicentrics and micronuclei ± 95% confidence limits. Dose-effect curves are linear-quadratic for dicentrics and micronuclei.

set up in cultures in the same manner and at the same time as the chromosome assay. After 48 h, cells were spun down and the medium and serum removed. Cells were washed twice in PBS and centrifuged at 300 *g* for 10 min at room temperature. The pellet was resuspended in approximately 500 µl of the remaining solution and kept frozen at − 70 °C before being used for the subcellular fractionation. The procedure of McCord and Fridovich [27] was used with minor modifications. Hemoglobin was removed by adding chloroform and ethanol [28] and after centrifugation for 10 min at 1200 *g*, SOD activity was measured by the method of Misra and Fridovich [29]. The autooxidation reaction of epinephrine to adrenochrome was performed in 3 ml of 0.05 M Na_2CO_3 at pH 10.2. Inhibition of autooxidation was monitored at 480 nm. After assaying total SOD activity, the samples were treated with 4 mM KCN to inhibit cytosolic SOD [30]. The values thus obtained and the differences between the two measurements were considered as MnSOD and CuZnSOD, respectively. The results were expressed in units of enzyme activity. Protein concentration was determined by the method of Lowry et al. [31].

Statistics. For analysis of the dose-effect relationship, the data were fitted by least-square regression analysis to a linear-quadratic model using the software package Origin 5.0 for Windows 9.5. The number of cells analyzed ranged from 111 to 876, more cells being examined at the lower dose ranges.

The correlation between the incidence of chromosome aberrations, MN, activities of MnSOD and CuZnSOD, and the proliferative ability of lymphocytes was evaluated using linear regression analysis (statistical software package 'Statistics,' for Windows 9.5, version 4.5). Correlation coefficients (R) were calculated at a significant level of $P < 0.05$.

Results

Experimental data for the yield of chromosome aberrations in cultured human lymphocytes as a function of absorbed dose were considered in three aberration groups. These were dicentrics (tri- and tetracentrics being scored as two and three dicentrics, respectively), sum of dicentrics and centric rings (exchanges), and excess acentrics. Chromosome and MN findings and measurement of SOD activities after proton irradiation in vitro are listed in table 1 together with the intercellular distribution of dicentrics and MN for each irradiated sample.

Dicentric as well as MN dose-responses fit a linear-quadratic form ($y = c + \alpha D + \beta D^2$) (fig. 1). With increasing dose, specific activities of both enzymes decreased: cytosolic SOD from 147.2 to 45.9 U/mg protein, MnSOD from 19.5 to 11 U/mg protein. Among irradiated samples, significantly lower proliferation ability was observed in those irradiated with 3 and 4 Gy against samples irradiated with 1 and 2 Gy.

The incidence of chromosome aberrations after X-ray irradiation in vitro, the distribution of dicentrics, MN incidence and distribution, as well as SOD activities are listed in table 2. Chromosomal and MN dose-responses fit a linear-quadratic form ($y = c + \alpha D + \beta D^2$). Values for spontaneous aberration frequencies were taken from historical laboratory controls consisting of 104 healthy donors with a total of 2894 cells analyzed. Obtained values of α and β for chromosomes and MN are presented in table 3. A significant increase in the specific activity of both SODs, particularly MnSOD, was recorded in samples irradiated with up to 1 Gy of X-rays, decreasing rapidly thereafter.

The proliferation potential of X-ray-irradiated lymphocytes was lower than that of controls. Among irradiated samples, a significantly lower proliferation ability was observed in samples irradiated with 3 and 4 Gy against samples irradiated with 1 and 2 Gy.

Correlations between all biological endpoints in irradiated samples are presented in table 4. An inverse, statistically significant correlation between the incidence of proton-induced aberrations and the activity of SODs was observed ($R = -0.81$, $P < 0.05$; $R = -90$, $P < 0.05$ for CuZnSOD and MnSOD, respectively). The activity of both SODs correlated positively with the proliferation potential of lymphocytes ($R = 0.93$, $P < 0.05$; $R = 0.92$, $P < 0.05$ for CuZnSOD and MnSOD, respectively). In samples irradiated with X-rays, no statistically significant correlation between the incidence of chromosome aberrations or MN and the activity of SOD was found ($R = 0.045$, $P > 0.05$). A positive, but statistically nonsignificant correlation between the activity of SODs and the proliferation potential of lymphocytes in X-ray-irradiated samples was observed ($R = 0.38$ and $R = 0.11$, respectively; $P > 0.05$).

Table 2. Dose-response (300 KeV X-rays, dose rate 1 Gy/min).

Chromosomes

Dose (Gy)	Number of cells scored	Dicentrics per cell (y)	Exchanges per cell	Excess acentrics per cell	Distribution of dicentrics between cells (number of cells with indicated number of dicentrics)			
					0	1	2	3
0	2894	0.001	0.001	0.001	2891	3		
0.5	876	0.045	0.057	0.070	838	37	1	
1	293	0.129	0.170	0.228	258	32	3	
2	282	0.354	0.431	0.531	194	76	12	
3	174	0.574	0.701	0.804	101	53	16	4
4	111	0.90	1.05	1.153	39	46	24	2

Micronuclei

Dose (Gy)	Number of BN cells scored	Number of micronuclei	MN/1000 BN cells	Micronuclei distribution between BN cells							Proliferation index (CBPI)
				0	1	2	3	4	5	6	
Control	1016	21	21	997	17	2					1.97
0.5	1477	100	68	1393	72	10	2				1.91
1	3112	505	148	2722	331	51	6	2			1.87
2	2471	971	393	1756	500	179	31	4	1		1.83
3	1469	1162	791	725	438	222	64	15	2	3	1.77
4	1007	1021	1014	314	296	225	119	35	11	7	1.74

SODs

Dose (Gy)	CuZnSOD (U/mg protein)	MnSOD (U/mg protein)
Control	141.24	15.3
0.5	144.48	21.5
1	168.19	29.85
2	113.91	18.35
3	131.97	19.05
4	132.29	16.25

Table 3. Dose-effect coefficients and goodness of fit for dicentric and micronuclei data fitted to the linear-quadratic model $y = \alpha D + \beta D^2$.

Radiation quality	C (constant)	$\alpha \pm$ SE	$\beta \pm$ SE	α protons/α X-rays
300 kVp X-rays (dicentrics)	-0.002 ± 0.004	0.094 ± 0.006	0.032 ± 0.001	1.26
22.6 MeV protons (dicentrics)	0.03 ± 0.004	0.119 ± 0.057	0.029 ± 0.014	
300 kVp X-rays (micronuclei)	-0.037 ± 0.024	0.138 ± 0.033	0.032 ± 0.008	0.59
22.6 MeV protons (micronuclei)	0.030 ± 0.031	0.082 ± 0.031	0.033 ± 0.007	

Table 4. Correlation between analyzed biological endpoints in irradiated samples.

	22.6 MeV protons		300 kVp X-rays	
	activity of CuZnSOD (U/mg protein)	activity of MnSOD (U/mg protein)	activity of CuZnSOD (U/mg protein)	activity of MnSOD (U/mg protein)
Incidence of dicentrics per cell	$R = -0.81$*	$R = -0.90$*	$R = -0.45$	$R = -0.35$
Incidence of micronuclei per cell	$R = -0.81$	$R = -0.89$*	$R = -0.45$	$R = -0.36$
Proliferation index (CBPI)	$R = 0.93$*	$R = 0.92$*	$R = 0.38$	$R = 0.11$

R = correlation coefficient (linear regression analysis), *$P < 0.05$

Table 5. RBE for dicentrics with 22.6 MeV protons.

Dose (Gy)	Incidence of proton-induced dicentrics	Incidence of X-ray-induced dicentrics	RBE
0.5	0.106	0.045	2.20
1	0.284	0.129	1.78
2	0.365	0.354	1.01
3	0.62	0.574	1.08
4	1.04	0.90	1.10

Protons were more effective than X-rays in producing dicentrics, particularly at 0.5 and 1 Gy, and were less effective in inducing MN, particularly at doses higher than 1 Gy. The efficiency of protons in inducing dicentrics and MN for the dose range from 1 to 4 Gy was calculated as the ratio of the two α coefficients (table 3). The relative biological efficiency (RBE) for dicentrics varied with dose, from 2.2 to 1.01 (table 5). Radiation-induced activity of both SODs, particularly MnSOD, was found only in X-ray-irradiated samples. In samples irradiated with protons, the activity of both SODs decreased rapidly with increasing radiation dose (table 2, figs 2, 3).

Discussion

Employing chromosome aberrations and MN as biological endpoints, we investigated the dose-effect relationship for 22.6 MeV protons and the contribution of intracellular SODs (cytosolic and mitochondrial) to the yield of radiation-induced aberrations. A linear-quadratic dose-response relationship for the production of dicentrics and MN in human lymphocytes was established in our study. The linear as well as quadratic coefficient obtained in this experiment with 22.6 MeV protons seems to be in accordance with previous estimations of dose-responses for human lymphocytes in vitro [32–35]. Statistics confirmed that the distribution of dicentrics among proton-irradiated lymphocytes is overdispersed compared to Poisson expectations, indicated by the relative variances (σ/y) of the distributions being larger than unity. At any observed mean aberration frequency, more cells with multiple aberrations and less

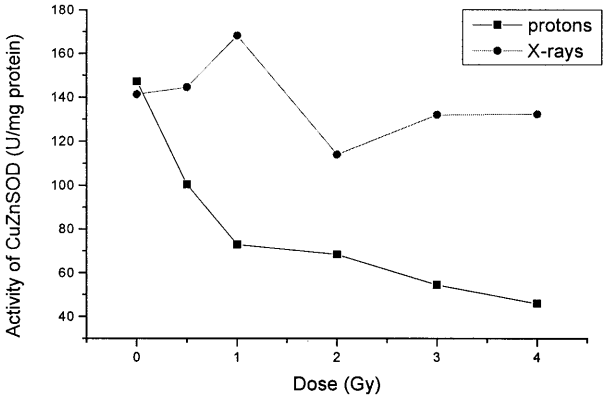

Figure 2. Dose dependence of CuZnSOD in X-ray- and proton-irradiated samples.

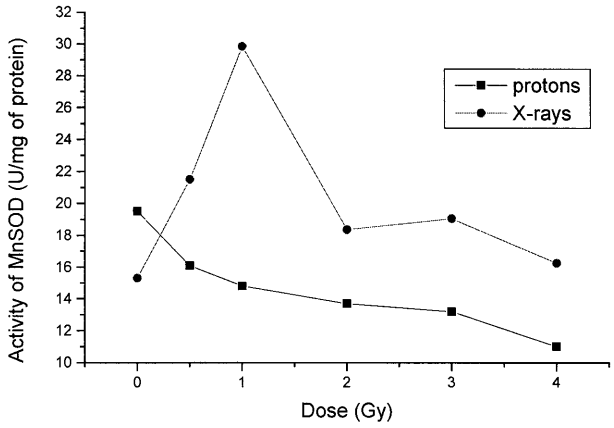

Figure 3. Dose dependence of MnSOD in X-ray- and proton-irradiated samples.

with one aberration, than expected from a Poisson distribution, were found. For each radiation dose, the U value was negative and statistically nonsignificant. The efficiency of protons in producing chromosomal aberrations (for doses ranging from 0.5 to 4 Gy) is presented as the ratio of α-coefficients of two radiations (α protons/α X-ray = 1.29). The RBE for dicentrics varies with dose: the highest (2.2) was recorded at 0.5 Gy, and the next highest at 1 Gy (1.78). At higher doses (2–4 Gy), the RBE for dicentrics ranged from 1.01 to 1.1. In contrast to X-ray-irradiated samples, where a significant increase in the specific activity of MnSOD was recorded, protons markedly reduced it. A suppressed antioxidative defense potential of leukocytes irradiated with protons, seen as a reduced activity of both SODs, particularly MnSOD, could be an important contributor to the RBE of protons.

Although numerous experiments have been performed to establish the RBE of charged particles, two main hypotheses have been presented to explain the increase in effectiveness of high-linear energy transfer (LET) radiation in killing cells. It has been suggested that as LET increases, the dsbs are formed close together within the cell increasing probability of interactions causing chromosomal exchanges [36, 37]. Ward [2] and Jostes et al. [38] postulate that repair of lesions determines the RBE of the radiation. However, radiation induces reactive oxygen species which are involved in the signal transduction pathway initiated by radiation, so various free radical scavengers can attenuate the response [39]. SODs play a key role in preventing the DNA damage caused by reactive free radicals, and SOD activity, particularly that of MnSOD depends on the dose and quality of radiation. The activity of CuZnSOD decreased significantly after irradiation with protons, but not with X-rays. CuZnSOD is a dimeric protein localized in the cytoplasm, which has higher concentrations of low-molecular-weight antioxidants than mitochondria, and milder changes in the levels of free radicals; enzyme function is therefore preserved more easily. On the other hand, MnSOD is a tetrameric protein, localized in the mitochondria, which are centers of cellular oxidoreduction processes, and are particularly sensitive to irradiation that might induce or suppress mitochondrial activity by direct or indirect effects [40]. Low-LET radiation induces increased activity of MnSOD that protects DNA against reactive oxidative species and possibly reduces the level of radiation-induced DNA damage. The mechanism is dose-dependent and might operate up to a radiation dose of 1 Gy; thereafter, the activity of MnSOD is markedly reduced. A linear-quadratic model, commonly used for fitting the dose-response, shows that a linear coefficient defines a slope of the curve up to doses of 1 Gy. At higher doses, MnSOD activity is further reduced, and the chromosomal dose-response becomes quadratic. Data are accumulating to indicate that free radicals, particularly superoxide anion radicals, present signal molecules, which can regulate the expression of antioxidant and repair enzymes and at the same time modulate the efficiency of antioxidative defense and repair of DNA [41, 42].

This study demonstrated that protons are more effective in producing exchanges and less effective in producing MN than X-rays. In samples irradiated with protons, some of the observed abnormalities could not be counted as MN because there were chromatin bridges between the main nucleus and the MN or because extremely small micronuclei (less than 1/10 of the main nucleus) were frequently observed. This probably explains the lower yield of proton-induced micronuclei in vitro. Thus, the beta term obtained from fitting MN data to the linear-quadratic model is significantly lower than those obtained for dicentrics. Two, not mutually exclusive, hypotheses could explain such an observation: that two or more aberrations in a cell might coalesce to produce fewer MN [43] or that with increasing radiation doses, the likelihood that MN could be included in one of the main daughter nuclei increases [44]. Uncertainty remains as to what objects become micronuclei after high-LET irradiation. Since the MN test enables measurement of the proliferation ability of the cells (CBPI), this assay was employed to estimate the radiation-induced mitotic delay in all irradiated samples. A statistically significant radiation-induced delay was observed only in samples irradiated with 3 and 4 Gy protons, a similar result to that found with X-rays.

Conclusion. Protons are more effective in producing dicentrics and are less effective in producing MN than X-rays. Irradiation with X-rays induces increased activity of MnSOD, whereas protons reduce the activity of both SODs markedly. As a consequence of the much reduced activity of MnSOD, the chromosomal dose-response changes from a linear to a quadratic curve. The study

demonstrated that the RBE for dicentrics with 22.6 MeV protons varies with dose (from 2.2 to 1.01) and that a reduced activity of MnSOD is an important contributor to the RBE of protons.

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