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EFFECTS OF TESTICULAR IRRADIATION ON STEM CELL SURVIVAL, HORMONAL ENVIRONMENT AND SPERMATOGENIC CELLS IN WISTAR RATS

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Abstract

Adult male Wistar rats were exposed to 3.0 Gy local testicular irradiation. Testes of irradiated and non-irradiated rats were examined histologically and flow cytometrically, at several intervals up to 78 days after irradiation. Concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone determined at these intervals were not different from those of controls. The survival of stem cells were measured 11 weeks after irradiation (with doses varying from 1.0 Gy to 6.0 Gy at 0.6 Gy/min) by means of the repopulation index and by the number of haploid cells (spermatids). Correlation between both methods and relation to stem cell survival were discussed. The dose response curves yielded D_0 values of stem cell survival of 2.33±0.06 Gy (repopulation index) and 2.08±0.08 Gy (number of haploid cells). The D_0 value of the rat was not much different from that found in mice. It was concluded that the used parameters can offer insight when studying hormonal substances during irradiation.

Key words: Radiobiology, testicular irradiation, rats, stem cells, hormonal environment, spermatogenic cells.

With the success of irradiation in prolonging the lives of patients with malignant diseases one started to realize that permanent gonadal damage may be induced. A treatment which could reduce the magnitude of testicular damage is highly desirable since it may result in preservation of reproductive function.

It is known (9, 10, 17, 29) that radioresistant stem cells play a crucial role in long term effects of irradiation on fertility. Shortly after testicular irradiation stem cell spermatogonia are almost the only spermatogonia present (1, 11). It was suggested (8, 29), that the sharp increase of stem cell radiosensitivity occurring shortly after irradiation, could be attributed to a change of surviving stem cells towards a much more radiosensitive phase of their cell cycle. From a theoretic point of view, hormones could be considered to be able to reduce or to avoid radiation damage, arresting stem cell division in the radioresistant phase. This could protect stem cells from depletion, resulting in preservation of reproductive function. While proof of this hypothesis is lacking, its validity can be tested by interrupting the pituitary-gonadal axis by hormones during testicular irradiation. There are a number of studies which have set precedent in terms of protection of spermatogenesis by hormones (6, 12, 13, 18, 21, 24). However, these studies have in general concentrated upon protection from cytotoxic drugs.

Before studying if hormonal treatment during irradiation is a possible mechanism to protect the testis from radiation damage, definition of parameters are necessary. The purpose of this investigation was to define the radiosensitivity of stem cells and to study the effect of irradiation on hormonal, histologic, and flow-cytometric parameters.

Material and Methods

The experiments were started with 3-month-old male outbred Wistar rats (Cpb:WU) each weighing about 300 g. The animals were allowed free access to dry pellet food (RMH-TM, Hope-Farms, Woerden, The Netherlands) and tap water. They were housed in groups of 4 in well ventilated cages at 22°C with a day/night rhythm of 12 hours. Twenty-four rats were used to measure the D_0

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value of stem cell survival. Groups of 4 rats each were irradiated with 0 (controls), 1.0, 2.0, 3.0, 4.0 and 6.0 Gy and killed 11 weeks after irradiation. Thirty-six Wistar rats were used to study the local effect of 3.0 Gy testicular irradiation dose and were divided into two groups consisting of 18 rats each. Group 1 was given one single testicular irradiation dose of 3.0 Gy, whereas Group 2 underwent the same procedure except no irradiation was given (controls). Serum hormone concentrations, number of tetraploid and haploid cells and testicular histology were quantified at day 0, 7, 14, 21, 28, 39, 52, 65, 78 after irradiation. Blood was collected by heart puncture and after centrifugation (1200 g, 10 min) serum samples were stored at −20°C until analysed. The testes were removed and weighed immediately after heart puncture.

Irradiation conditions. The rats were anaesthetized with an Alyrane (2-chloor-1,1,2-trifluorethyl-diflour methylether) oxygen gas mixture and irradiated in prone position with the 18 MV photon beam of a Saturne linear accelerator (CGR, Buc, France). The Saturne is equipped with a mercury shielded irregular field system device (2) which was used to define 4 irradiation portals of 4 cm × 5 cm each, so that 4 rats could be irradiated on their scrotum simultaneously. To achieve a dose homogeneity in the target volume within 10 per cent it was necessary to get rid of the build region in the beam. Therefore a 1.5 cm thick plexiglass plate was positioned just above the rats. The dose rate during the experiments was 0.6 Gy/min.

Stem cell survival assays. Spermatogonial stem cell survival was evaluated by the repopulation index (RI) and by counting the number of haploid cells (spermatids) by using flow cytometry. The left testis from each killed animal was fixed in Bouin's fluid and embedded in paraffin wax. From each testis three sections, separated from each other by a distance of 500 μm, were taken from the midportion of the testis and stained with Periodic acid-Schiff (PAS). The percentage of tubules which showed spermatogenic repopulation in histologic sections, 11 weeks after irradiation, was defined as the repopulation index (7). A tubulus was considered to repopulate when it contained at least one spermatogonium. The right testis was used for flow cytometric analysis. Radiosensitivity of the stem cell can be estimated by the dose required to reduce survival to 37 per cent of the initial value on the straight-line portion of a survival curve (D_0 value). The D_0 value of the curve was calculated with a linear regression model by a simple least-square analysis at the mean RI values as was previously described (7).

Histology. The left testis from each animal was fixed in Bouin's fluid, dehydrated in 70 per cent ethanol and embedded in paraffin wax. Duplicate transverse sections of 5 μm were taken from the midportion of the testis and stained with PAS with and without prior diastase digestion. For histologic measurement, the germ cell maturation sequence of 14 stages as proposed by LEBLOND & CLERMONT (20), was used. The counts were made on cross sections of 25 seminiferous tubules. The numbers of spermatogonia or preleptotene spermatocytes were expressed as numbers per 100 Sertoli cells. Expressing the data in this way corrects for tissue shrinkage and permits a comparison of germ cell counts from one time interval to another. Spermatogonia of types A 0 and A 1 were scored as a single class of cells since in our material they could not be differentiated. Type B spermatogonia were scored at stage 6, intermediate spermatogonia at stage 3 and preleptotene spermatocytes at stage 8 of the cycle.

Flow cytometry. The right testis from each animal was dissected free from fat and connective tissue. To obtain a fluorescent stained single cell suspension, preparation and staining procedure was carried out as previously described (25). Haploid and tetraploid cells were measured by flow cytometric analysis which was performed on an Ortho System 50-H (Ortho Instruments, Westwood, Massachusetts, USA) using the 457 nm line of the argon ion laser (Spectra Physics, Mountain View, California, USA) at 200 mW power.

Hormone measurements. Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by double-antibody solid phase radioimmuno assays using the rat gonadotrophin kit reagents supplied by NIADDK Bethesda, Maryland, USA. The measurements were expressed as μg/l in terms of the NIADDK-rat FSH-RP-1 and LH-RP-1 standards respectively. The minimum detectable concentrations were 10 μg LH/l and 50 μg FSH/l. The precision was 5.9 per cent within assays and 6.7 per cent between assays for duplicate measurements of FSH in a serum pool (mean value 264 μg/l) in 15 consecutive assays. For LH these values were 3.9 per cent and 9.6 per cent respectively (mean: 59 μg/l) in 15 consecutive assays. Testosterone was measured by a dextran-coated charcoal radioimmuno assay after extraction of serum samples with diethyl ether. The antiserum used was raised in a rabbit and directed against testosterone-3-(0-carboxymethyl)-oxime-BSA. The sensitivity of the assay was 0.1 nmol/l and the precision, as calculated from a serum pool (mean value: 1.85 nmol/l) after 14 consecutive measurements), was 3.9 per cent within assays and 5.7 per cent between assays.

Statistical analysis was performed by using the two-sided Mann-Whitney U-test. Differences were considered to be statistically significant when $p<0.05$.

Results

A semilogarithmic plot of the percentage of stem cell survival versus radiation exposure was determined from the RI (Fig. 1) and from the number of haploid cells (Fig. 2). In view of the better multiple correlation coefficient, the data point of 1.0 Gy was excluded. The regression analysis, without the 1.0 Gy data points yielded (mean ± SD) D_0 values of 2.33±0.06 Gy (R1) and 2.08±0.08 Gy (haploid cells). The logarithm of the RI was a linear func-

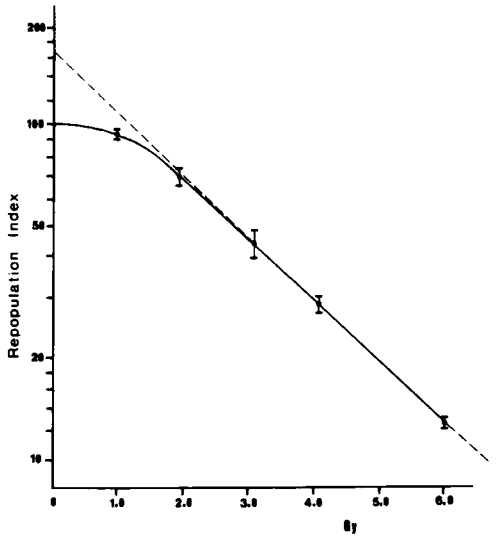

Fig. 1. Stem cell survival measured by the repopulation index 11 weeks after irradiation with different dosages (mean ± SD of 4 rats). D_0=2.33±0.06 Gy.

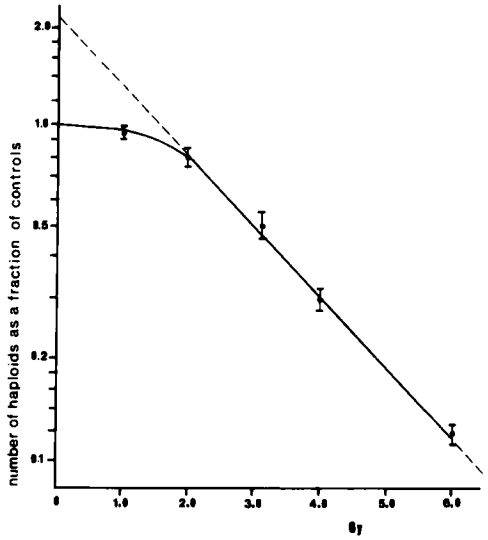

Fig. 2. Stem cell survival measured by the number of haploid cells 11 weeks after irradiation with different dosages (mean ± SD of 4 rats). D_0=2.08±0.08 Gy.

tion of dose (multiple correlation coefficient >0.95). The data points of both methods (expressed as fractions of controls) were not significantly ($p<0.05$) different from each other. The n value (extrapolation number) was 167 (RI) and 2.12 (number of haploids as a fraction of controls).

The effect of 3.0 Gy single dose irradiation on testes weight, the number of spermatogonia and preleptotene spermatocytes and on the serum concentrations of FSH, LH and testosterone are presented in the Table. Testes weight declined to 50 per cent of those of control value at day 39. As a result of this irradiation dose a large fraction of type A, intermediate, and type B spermatogonia and preleptotene spermatocytes were rapidly destroyed (Table). Recovery was seen from day 14 onwards. The numbers of type intermediate spermatogonia showed an overshoot phenomenon. The elimination of spermatogonia resulted in a progressive disappearance of the numbers of tetraploid and haploid cells. The total number of spermatids dropped to 10 per cent of the control value. It can be seen that recovery of spermatids after 3.0 Gy local irradiation commenced after 52 days. The concentrations of FSH, LH and testosterone were equal to that of controls at each time interval.

Discussion

Two assays for testicular stem cell survival have been applied to obtain survival curves. The number of clones in the testis found several weeks after irradiation have been described (7, 28) as a direct measurement of stem cell survival. Since there is also a quantitative correlation (22) between testicular stem cell survival and sperm production and since haploid cells are corresponding only to spermatids and to no other testicular cells, the number of haploid cells as measured by flow cytometry can also be used as a measurement of stem cell survival (14). The relationship between spermatid production and stem cell number may by linear. However, for the mouse, Meistrich (22) has demonstrated that although sperm production and stem cell survival as measured by RI were highly correlated the relationship was not linear (the regression line had a slope of 1.7 on a log versus log plot).

The evaluation of the stem cell survival curves shows a good parallel between both stem cell assays in the actual dose range. Our D_0 values are, however, in contrast with those of others. Clow & Gilette (3) and Erickson (10), found D_0 values of 3.80 Gy and 3.73 Gy respectively. A comparison of the reported D_0 values for mice in literature by De Ruiter-Bootsma et coll. (8) and Withers et coll. (29), indicates that our D_0 value of the rat is not much different from that found in the mouse (2.4 Gy and 1.8 Gy respectively). This is in accordance with the fact that the dose required to produce infertility (15, 19) in the mouse is the same as that in the rat. However, in the low dose range when the repopulation index exceeds the value of 0.5, the assay reliability is controversial as multicellular origin of colonies cannot be excluded. Correction must therefore be made for the possibility of clone confluence (23). If so, the corrected mean D_0 value of the RI values was even lower (1.8 Gy).

Since spermatogonia are so very radiosensitive (1, 9) a maturation depletion occurs of other spermatogenic cells in their order of developmental sequence, eventually resulting in loss of sperm. This is clearly shown by the decrease in numbers of preleptotene and pachytene sper-

Table

Effect of a single dose irradiation of 3.0 Gy on (mean ± range) testicular weight (A), on the numbers per 100 Sertoli cells of type A (B), intermediate (C) and B spermatogonia (D) and preleptotene spermatocytes (E), on the number of haploid cells (F; spermatids), on the number of tetraploid cells (G; mainly pachytene spermatocytes), and on serum hormone concentrations of FSH (H), LH (I) and testosterone (J) in adult rats

	Days								
	0	7	14	21	28	39	52	65	78
A (g/kg)	9.47	8.67	8.34	7.12	6.91	4.36	4.22	5.37	6.75
	9.93	9.40	8.41	7.31	6.49	4.49	4.74	6.33	6.95
	10.39	10.12	8.47	7.49	6.06	4.62	5.26	7.29	7.15
B	8	1	1	1	1	1	4	3	6
	10	2	1	2	2	4	6	9	8
	12	3	1	3	3	7	8	12	10
C	55	10	10	15	20	35	54	64	50
	60	12	14	18	24	38	57	66	53
	65	14	18	21	28	41	60	68	56
D	75	36	8	4	15	47	57	55	64
	90	45	18	9	22	55	62	61	72
	105	54	28	14	29	63	67	67	80
E	140	22	4	4	6	46	106	119	122
	150	30	8	7	15	57	116	128	129
	160	38	12	10	24	68	126	137	136
F ($\times 10^6$)									
	71.6	77.8	51.1	–	37.4	3.4	3.8	45.3	46.7
	75.0	78.2	55.3	–	44.4	4.5	3.9	45.4	49.0
	78.4	78.6	59.5	–	51.4	5.6	4.0	45.5	51.3
G ($\times 10^6$)									
	5.58	6.64	5.93	4.28	2.65	1.00	3.39	4.65	3.54
	5.70	6.93	6.44	5.08	3.25	1.33	3.51	4.85	3.66
	5.82	7.22	6.95	5.88	3.85	1.66	3.63	5.05	3.78
H (μg/l)	150	150	175	140	140	170	165	155	155
	160	175	190	160	160	190	185	170	165
	170	200	205	180	180	210	205	185	175
I (μg/l)	28	35	28	24	27	31	28	37	29
	32	38	34	26	32	33	32	38	33
	36	41	40	28	37	35	36	39	37
J (nmol/l)	10	6	8	7	8	7	7	6	8
	14	9	10	9	10	9	10	9	10
	18	12	13	11	12	11	13	12	12

matocytes (the numbers of tetraploid cells are mainly consisting of pachytene spermatocytes (26)) and by the low numbers of spermatids (number of hapoid cells) from day 39 till day 52. This decrease in the number of pachytene spermatocytes and spermatids was not paralleled by an alteration in serum hormone concentrations. Studies (5, 16) showed an increase in serum FSH depending on the used dose of irradiation. Conflicting results have, however, been reported after a radiation dose of 3.0 Gy. CUNNINGHAM & HUCKINS (4) and VERJANS & EIK-NES (27) showed no alteration in FSH levels, whereas HOPKINSON et coll. (16) did. Our findings agree with the findings of CUNNINGHAM & HUCKINS (4), that the selective abscence of spermatogonia and spermatocytes do not affect serum hormone concentrations. From our results we conclude that the used parameters offer us insight when studying hormonal substances during irradiation. This will be the subject of a subsequent report.

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