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MORPHOMETRICS OF CELLULAR DAMAGE IN MICE TESTIS
RECEIVING X-RAY AND HIGH-ENERGY PARTICLE IRRADIATION

Walter J. Sapp
Principal Investigator
Department of Biology
Tuskegee University

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Introduction

Very shortly after the discoveries of the various types of radiation, it was realized that drastic changes could be induced in tissues with irradiation. These changes are now known to significantly damage nucleic acids and thusly genetic expression at several levels of cellular activity, including death of cells.

Since the advent of manned space flight in the early 1960's, concern for the well-being of space travellers has emerged as an important consideration. This is particularly true now since the successful flights of the space shuttle portend a time of increased numbers of space travellers. Space stations will provide for longer tours of duty and will be higher than shuttle to avoid any atmospheric drag. The possibility also exists for duty tours in geosynchronous orbit where the radiation dose is estimated to be 40 rem/90 days. This is four times the allowable dose on Earth. This "cosmic" irradiation is mainly characterized by atomic nuclei stripped of their electrons (HZE particles). There is concern not only for the exposure on space flights, but also for the long-term effects which could manifest themselves later.

Since radiation effects are particularly apparent in a rapidly proliferating tissue, the seminiferous epithelium in mammals provides an excellent model for studies in this area. Through the years, the mouse has been one of the most widely used animals in studies of spermatogenesis under both normal and experimental conditions (1, 2, 3, 4, 5, 6, 7). The work of Oakberg and Huckins has been particularly noteworthy in the establishment of a model for rodent spermatogenesis and the subsequent effects of radiation on the various cell types involved (8, 9, 10, 11, 12, 13, 14, 15).

It has been shown that within two to three days after irradiation (150

rad) the germinal epithelium is devoid of most spermatogonia except for some lingering A cells and the radio-resistant A stem cells (13, 14, 16). These cells will eventually repopulate the seminiferous epithelium. While results with x-ray and other low energy irradiation have been well documented (6, 18, 19, 20), the comparable effects of high energy (LET) irradiation similar to the cosmic type (HZE particles) have received only limited attention, particularly at low dose levels exposures. Alpen and Powers-Risius (21) have shown a dose dependent overall weight loss in testis after exposure to heavy particle beams of argon and neon; however, their work did not include cytological correlation of epithelial cell loss with general weight loss.

The present investigation was designed to test the effects of low dose, high LET, HZE particles on the cytology of murine seminiferous epithelium and to report the relative biological effects (RBE) of HZE particles at low dose levels and to compare these to radiation effects of low LET sources, such as x-ray.

Methods and Materials

Male B₆ D₂ f₁ mice 12-17 weeks old were used as experimental animals in this study. Animals were maintained in cages and were given food and water ad libitum. Prior to radiation exposure, animals were anesthetized with sodium pentobarbital. Control mice, although not irradiated, were also anesthetized. All irradiation was administered as single doses. Relatively high dose rates were utilized, assuring a short exposure time and no variation in results as a function of dose rate (22).

A Philips orthovoltage generator was used for x-irradiation; doses were from 0.01 to 1 Gy at 225 keV and 15 mA; half value was 0.35 mm Cu and dose rates were between 0.50 and 0.60 Gy per minute. Helium ions were produced at the Berkeley 184 in. synchrocyclotron with initial energies of the particles at 228MeV/amu at a dose rate of 1.5 Gy per minute. Argon heavy ion beams were produced at the Berkeley Bevalac. Mice were irradiated with the plateau region of the beam (570MeV/amu) at dose rates of 0.25, 0.59 or 2.00 Gy per minute. Details on the radiation sources and methods of exposure for helium and argon have been reported by Curtis, et al (23). The beam was collimated so as to expose the lower half of the mice which were irradiated in a frontal position.

Animals were anesthetized seventy-two hours post-irradiation and perfused in toto with triple fix (24). Testes were removed and embedded in Epon Araldite using standard procedures. Thick sections (2um) were cut on a DuPont Sorvall MT2B ultramicrotome and stained with either 0.1% Toluidine Blue O in 1% sodium borate or with ammoniacal silver (25). Thin sections (60nm) were cut and stained with conventional techniques and examined with either a Philips 201 or 300 electron microscope at 60kv. At least four animals were used at each radiation dose level. Tubule cross sections were collected from both right and left testes of each animal.

Seminiferous tubule cross sections were staged according to spermatogonial configuration. Essentially, this scheme is based on six generations of spermatogonia as they progress from A_1 to the B type (26). In addition, spermatid characteristics, such as acrosomal development, were used to verify tubule classifications. Spermatogonial cell types were identified based primarily upon nuclear morphology. Consequently, only cells with nuclear profiles visible within the section plane were identified and counted.

Counts of the surviving spermatogonia were scored at tubule Stage 6 and at tubule Stage 1. Stage 6 contains the lowest portion of surviving spermatogonia at three days post-irradiation. Scoring at this stage is advantageous because the stage and the cell types present are easily identified. The spermatogonia found in Stage 6 at seventy-two hours post-irradiation are progeny of cells that were A_3 , A_4 , A_5 or A_6 cells at zero time, the time of the radiation event. By assaying this stage, we are reporting the radio-sensitivity of this group of cell types. Likewise, counts of surviving spermatogonia and preleptotene spermatocytes in Stage 1 tubules at 72 hours post-irradiation are indicative of the radiosensitivity of Intermediate and Type B spermatogonia. Stage 6 data are expressed as the mean total of all surviving spermatogonia, regardless of type, observed per tubule cross section. Stage 1 data reflect the mean total of only pre-leptotene spermatocytes observed per tubule cross section. In addition, cell counts are expressed as "survival fraction" (S/S_0) based on cellular populations in the control testes. Differences between levels of spermatogonial survival were determined by an unpaired Student's t-test ($P < 0.05$).

Results

Since the tissues are fixed and embedded by methods used for electron microscopy, it is possible to obtain thin sections for ultrastructural analysis in addition to the thick sections suitable for light microscopy. Figure 1, is a light micrograph of control unirradiated tissue, both from a Stage 6 tubule. The nuclei of spermatogonial cells can be seen along the periphery of the seminiferous tubule near the basement membrane. The nuclei of Type B spermatogonia are most numerous and appear uniformly spherical. They are approximately eight micrometers in diameter and have characteristic large, dense, irregular heterochromatin granules. Less differentiated spermatogonia, the Type A cells, have larger nuclei, approximating twelve to fifteen micrometers, with an elliptical shape. The chromatin pattern in these cells is much finer, with small irregular granules appearing near the periphery at the nuclear envelope.

The loss of spermatogonia due to irradiation is readily apparent. Figure 2 represents a section of Stage 6 tubule 72 hours after 0.80 Gy of He irradiation. It is evident that killed and damaged cells are rapidly removed post-irradiation since virtually all Type B spermatogonia are absent; only Sertoli cells and stem cell (As) spermatogonia remain. Large pachytene nuclei of the primary spermatocytes now occupy the tubular periphery to a great extent.

Table 1 summarizes spermatogonial cell count data from all dose levels of each type of radiation. At radiation levels of 0.10 Gy and less, Type A spermatogonial response is similar, regardless of type of radiation, as reflected by spermatogonial survival in Stage 6 tubules 72 hours post-irradiation. Approximately one-fourth of all spermatogonial cells are lost at this dose level. In fact, even at the very low level of 0.01 Gy about 5% of the spermatogonia are killed. At slightly higher doses, spermatogonial response

begins to differ with type of radiation. These differences are made more apparent when the surviving fractions (S/S_0) of spermatogonia are expressed graphically (Figs. 4, 5, and 6).

Spermatogonia also demonstrate "tri-phasic" response when exposed to x-irradiation (Fig. 4). About 30% of the total spermatogonial population is lost with 0.10 Gy exposure providing a sharp decline in surviving fraction. However, with exposures from 0.1 Gy to 0.8 Gy the decline in survival fraction is much more gradual. Approximately an additional 40% of the spermatogonial population is lost within this range of exposures. Thirty percent of the total population remains intact, even at 0.8Gy. However, between 0.8 Gy and 1 Gy there is a very sharp decline in cell survival with only about 9% of the spermatogonia surviving the radiation event. Exposure to helium ions generates an almost linear decline in survival fraction until a dose level of 0.80 Gy is reached at which time there is a sharp decline (Fig.5). This contrasts dramatically with the spermatogonial response to argon irradiation (Fig. 6).

In argon treated tissue, an initial decline in survival fraction continues to the level of 0.15 Gy, then levels off slightly between 0.15 and 0.30 Gy. Between 0.30 Gy and 0.45 Gy there is a sharp decline in number of surviving spermatogonia with only about 11% of the total population remaining after exposure to 0.45 Gy (Fig. 6).

Table 2 summarizes the counts of surviving pre-leptotene spermatocytes in Stage 1 tubules. These cells are surviving progeny of Type B spermatogonia irradiated 72 hours earlier. Helium and x-ray exposure elicit a similar response in the testicular epithelium at dose levels less than 0.1 Gy. There is an approximate loss of 13% of the pre-leptotene spermatocyte population at 0.1 Gy of either helium ions or x-rays. In contrast, exposure to argon ions produces a much more deleterious effect with a loss of almost 20% of the

population at the very low dose of 0.05 Gy. At slightly higher radiation levels, 0.20 Gy for helium ions and x-rays and 0.15 Gy for argon ions, there is a very sharp decrease in spermatocyte survival. Comparison of the effectiveness of each of the types of radiation becomes apparent when surviving spermatocyte fraction (S/So) compared to control is expressed graphically (Figs. 4, 5, and 6). Argon exposure, a high LET ion, kills slightly more than 50% of the population at a dose level of 0.15 Gy while at 0.45 Gy only 4% of the spermatocyte population remains (Fig.6).

Response to irradiation is best compared when expressed as Relative Biological Effectiveness (RBE) where a given radiation response is compared to a response of an equal dose level of x-rays. Examination of Figures 5 and 6 reveals that the RBE's for helium and argon are low (i. e., response is similar to x-ray exposure) at low levels of radiation while the RBE for argon increases dramatically above exposure to 0.30 Gy. For example, at 0.05 Gy the RBE's for helium and argon are about 1. At 0.45 Gy argon exposure, the RBE becomes about 4.3.

An alternative measure of radiation effectiveness is D_0 , the dose needed to kill 37% of a given cellular population. Using Figures 4, 5, and 6 as examples, the D_0 's for x-ray, helium and argon are , , and respectively. These are the radiation levels required to decrease the pre-leptotene spermatocyte population by 37%.

It is interesting to note that counts of stem cell (A_s) spermatogonia showed very little change with exposure to radiation. Table 3 summarizes the numbers of surviving A_s spermatogonia in Stage 1 tubules. Decreases in cellular populations were noted only at high levels of radiation exposure, demonstrating the radioresistance of the stem cell spermatogonia.

Discussion

Utilizing tissue which had been prepared for both electron microscopic and light microscopic observation provided an opportunity for the correlation of cell count data (i.e., the dose related decrease in spermatogonial cell numbers) with the ultrastructure of the seminiferous epithelium (Philpott, et al. 1983). In addition, the excellent fixation quality and thinness of the epon sections make accurate identification of these cell types at particular stages possible (Chakrabarty, et al., 1984). Generally, there was a progressive increase in the number and size of heterochromatin granules in the nuclei of the Type A cells as they matured from undifferentiated A_s stem cells to the more differentiated Type A₄ cells. This change in morphology is characteristic of maturing spermatogonia and has been described in detail by Huckins (8). Nuclei of the Sertoli cells are also located along the periphery of the tubule but they are easily distinguished from germinal nuclei because of their fine chromatin pattern and distinct nucleolus.

All counts of surviving cells were taken from epon sections approximately 2µm thick. Since nuclear position and configuration are the most reliable way to distinguish the various types of spermatogonia present, only cells with visible and identifiable nuclear profiles in the section plane were counted. The number of cells (nuclei) present in any given section is, therefore, limited to an extent by the section thickness. This becomes evident when control (non-irradiated) cell populations are compared in Stage 1 and Stage 6 tubules. It is well documented (8,11) that in both rats and mice there are twice as many pre-leptotene spermatocytes present at Stage 1 than there were Type B spermatogonia at Stage 6 due to cell division. This difference is not apparent in our work because of the relative thinness of the sections. When thicker sections of control tissue, i.e., 6µm, (the thickness commonly used in

routine histological work), were analyzed, counts of cells (nuclei) present increased at least 30%.

Exposure to very low doses of either low energy or HZE radiations caused statistically significant ($P < 0.05$) decreases in cell survival. This indicates the presence of highly sensitive cells (Table 1) as well as less sensitive cells. The heterogeneous radio-sensitivities of the developmental types of spermatogonia in the mouse, and the high radio-sensitivity of certain Type A cells at low doses of radiation similar to the level of sensitivity of Type B cells, have been well documented, particularly in the early work of Oakberg (15, 16, 17, 27). Suci (1982), working with various mouse tissues, although not with the testis, postulated that the relative amounts of non-histone protein present in the nuclei of cells was correlated to the radio-sensitivities of the cells. He cited evidence that cells (nuclei) with large amounts of tightly bound non-histone protein, such as liver and kidney, are more radio-resistant than are lymphoid cells which contain relatively small amounts of bound non-histone protein. This heterogeneity in radio-sensitivity is also, at least in part, a result of the duration of the radio-sensitive "interphase" period of the cell cycle (6). The lengths of both the S phase and the G_1 phase of the cell cycle vary with the type of spermatogonia observed (28). The length of the DNA synthesis period is short in the radio-resistant Type A cells and becomes progressively longer as maturation and differentiation occur through the Type A cells to the Type B cells (6). An increase in radio-sensitivity with the lengthening of the S phase has been a consistent observation.

It should be noted that when surviving progeny of a single cell type (Type B spermatogonia) are assayed by counting Stage 1 pre-leptotene spermatocytes 72 hours post-irradiation, the survival curves are very different

(Figures 4, 5, and 6). The "triphasic" nature of the survival plot, indicative of a heterogeneous population, is not present. After an initial shoulder at low doses, the decline in cell survival is virtually linear. The presence of this shoulder is usually interpreted to indicate the ability of a cell or tissue to "repair" itself after low-dose radiation exposure (). This concept is particularly intriguing when the size of the shoulders obtained with different types of radiation are compared (Figures 4, 5, and 6). X-ray, a low energy radiation exposure, apparently is not as deleterious to Type B spermatogonia as is helium ion exposure, as evidenced by the large shoulder on the surviving spermatocyte plot. Argon exposure, on the other hand, provides virtually no chance for repair in these very sensitive cells and there is, as a result, little or no shoulder on the curve. This response should not be attributed only to the LET of the argon particle irradiation. It has been clearly demonstrated (21) that with heavy ions, the effectiveness of the radiation depends to a great extent on the mass of the particle also.

Although not quantitated in Stage 6 counts of surviving spermatogonia, it was observed that as radiation doses increased, Type B cells (surviving progeny of A_4 cells at the time of the radiation event) disappear first. This reflects a relatively high radio-sensitivity of A_4 spermatogonia. At higher doses only the radioresistant stem cells (A_s) remain.

Irradiation with either x-ray, helium or argon at doses less than 0.01 Gy produces similar levels of cell death indicating that the RBE's for these types of radiation at low doses are similar despite their differences in LET and atomic mass. The extremely radio-sensitive cells which are destroyed at these low dose levels appear to be uniformly sensitive to all types of radiation. This is supported when survival fractions of pre-leptotene

spermatocytes are observed in Stage 1 tubules. Curves are generally similar in shape at low doses, less than 0.10 Gy. At doses above 0.10 Gy, it becomes possible to see differences in cellular sensitivity to radiation type.

Presumably, the Type B spermatogonia, whose progeny are counted in this stage, are one of the most radiosensitive cells in the seminiferous epithelium and account for a large portion of the population decrease seen at low doses.

At higher dose levels, above 0.10 Gy, the greater effectiveness of argon is quite apparent in the dramatic decrease in the number of cells surviving the radiation event. One would expect these results because of the large atomic mass of argon compared to x-ray and helium. However, it should be noted that the relationship of particle mass, LET and RBE for spermatogonial killing is still not completely understood (21) and further studies at low energy levels and at low doses are needed to more fully elucidate the mechanisms involved.

The survival curves obtained for helium and x-ray are similar over the wide range of doses used. Helium particles produce effects similar to those generated by low energy radiation, such as x-ray or gamma rays (17) rather than the effects of high Z (particle) irradiation. This phenomenon has also been observed with intestinal crypt cell survival (22) where, in fact, helium was shown to be less effective than x-ray in eliciting a radiation response.

A very large portion of radiation work has been done utilizing high doses, 1 Gy and higher. These high doses in many ways mask the responses of the smaller individual populations of cells within a complex tissue, such as the seminiferous epithelium. This work, involving doses at very low levels of 0.01 to 1 Gy, has produced survival curves somewhat dissimilar to those in the literature. The curves generated in our experiments show an initial sharp decline in cell population at very low doses (a loss of approximately 10% of the

cells at less than 0.1 Gy). Most previous work describes a "shoulder" on the survival curve, particularly for low-energy irradiation such as x-ray (29). However, it should be pointed out that most of these experiments have utilized doses of 1.0 Gray or higher. In addition, at least one group (Ekstrand, et al . 1982) has expressed concern in using certain models of survival curves in radiation work, particularly with respect to the shoulder obtained at low doses. Using highly purified populations of lymphocytes, they obtained a biphasic curve and, as a result, concluded that one could not rely on the shape of the curve to predict subpopulations of cells with different radiation sensitivities. We are suggesting that our work at low doses has provided a refinement of previous work and has permitted the visualization of the heterogeneous cell population of the seminiferous tubules in its response to both HZE and low energy radiation.

The various responses of these different spermatogonial cell types, even at these very low radiation levels, are reflected in the changing slope of the survival curve over the increasing range of radiation dose. We are postulating that it is not useful, particularly at low dose levels, to pool all spermatogonia and discuss a D_0 for seminiferous epithelium in general. Rather, our data suggest there are separate D_0 values for individual spermatogonial cell types (A_{1-4} , In, B, etc.). Investigation of the irradiation response of each cell type can be accomplished by studying each of the six spermatogonial stages. For example, the Stage 1 counts of surviving pre-leptotene spermatocytes are an assay of the radio-sensitvity of Type B spermatogonia.

S U M M A R Y

Murine testes were exposed to single, low doses (less than 1 Gy) of either x-ray, helium, or argon radiation. Animals were sacrificed seventy-two hours later. Testes were fixed for transmission electron microscopy (TEM) and sectioned at either 60nm for TEM observation or at 2um for counting using routine light microscope methods. Counts of the total population of surviving spermatogonia, including all Type A cells, intermediate and Type B cells, were taken from tubule cross sections identified as Stage 6 and Stage 1 according to spermatogonial configuration. The surviving fraction of spermatogonia as compared to control, S/S_0 , was calculated for each dose. For both ions and x-rays, there was a rapid decline in survival at dose levels of .10 to .15 Gy in Stage 6 tubules. This was followed by a more gradual decrease in population. At higher doses, 0.30 Gy for argon and 0.80 Gy for helium and x-rays, the cell survival rates declined rapidly. Pre-leptotene spermatocytes in Stage 1 tubules exhibited a different survival curve indicating the extreme radio-sensitivity of Type B spermatogonia. Data verify that the seminiferous tubules are composed of a heterogeneous population of cells with different radio-sensitivities and that these differences are manifested even at very low doses.

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Table I. Counts of surviving spermatogonia in Stage 6 tubules after exposure to various doses of different types of irradiation.^a

Dose	X-RAY 50-60 Rads/Min			HELIUM (Plateau) 150 Rads/Min			ARGON (Plateau) 25-200 Rads/Min		
	Section Counted	Mean \pm S.D.	S/S ₀	Section Counted	Mean \pm S.D.	S/S ₀	Section Counted	Mean \pm S.D.	S/S ₀
C	684	30.20 \pm 2.69	1.00	1462	29.44 \pm 1.22	1.00	400	29.22 \pm 1.51	1.00
1	287	27.87 \pm 1.75	.96	624	27.97 \pm 1.33	.95			
5	309	25.63 \pm 2.84	.84	456	24.07 \pm 2.53	.82	400	24.43 \pm 1.42	.84
10	694	21.89 \pm 2.20	.72	1520	22.86 \pm 2.42	.78			
15							564	17.99 \pm 2.37	.62
20	439	18.41 \pm 2.90	.61	988	18.36 \pm 4.01	.62			
30							200	15.86 \pm 1.64	.54
40	573	14.91 \pm 2.50	.49	1308	13.21 \pm 2.28	.45			
45							400	3.19 \pm 1.07	.11
60	588	11.81 \pm 2.24	.39	160	9.26 \pm 1.25	.31			
80	775	9.40 \pm 4.15	.31	1256	6.26 \pm 2.19	.21			
100	592	2.83 \pm 1.11	.09	348	3.18 \pm 1.11	.11			

^a All means significantly (P<0.05) from control, unpaired t-test.

TABLE II

Surviving fraction of pre-leptotene spermatocytes in Stage I tubules after exposure to various levels of irradiation

	X-ray ^a	S/So	Helium ^a	S/So	Argon ^a	S/So
C	30.48 ± 1.72	1.00	29.69 ± 1.06	1.00	30.09 ± 2.76	1.00
1	28.61 ± 1.28	0.94	28.79 ± 1.93	0.97		
5	27.34 ± 2.18	0.90	26.43 ± 2.14	0.89	24.12 ± 3.01	0.81
10	26.62 ± 1.84	0.87	25.83 ± 3.01	0.87		
15					14.81 ± 2.31	0.49
20	23.49 ± 2.63	0.77	15.02 ± 2.76	0.51		
30			7.62 ± 1.34	0.23	4.51 ± 1.89	0.15
40	4.62 ± 3.71	0.15	2.95 ± 1.47	0.10		
45					1.23 ± 0.76	0.04
60	1.84 ± 2.26	0.06	1.48 ± 0.80	0.05		
80	0.52 ± 0.97	0.02	0	0		

^a Mean ± S.D., 500 frames counted. All means significantly (P<0.05) different from control, unpaired t-test.

TABLE III

Surviving fraction of spermatogonial stem (A_3) cells after exposure to various levels of irradiation.

	X-ray ^a	S/So	Helium ^a	S/So	Argon ^a	S/So
C	1.33 ± 0.79	1.00	1.68 ± 0.67	1.00	1.74 ± 0.81	
1	1.31 ± 0.71	0.98	1.35 ± 0.75	0.80		
5	1.44 ± 0.77	1.08	1.67 ± 0.80	0.99	1.64 ± 0.72	0.94
10	1.22 ± 0.75	0.92	1.55 ± 0.63	0.92		
15					1.61 ± 0.65	0.92
20	1.25 ± 0.70	0.94	1.63 ± 0.58	0.97		
30					1.17 ± 0.59	0.67
40	1.12 ± 0.76	0.84	1.73 ± 0.85	1.03		
45					0.73 ± 0.35	0.42
60	1.22 ± 0.79	0.92	1.36 ± 0.62	0.81		
80	1.08 ± 0.73	0.81	1.22 ± 0.61	0.73		
100	0.85 ± 0.64	0.64	0.54 ± 0.39	0.32		

^a Mean ± S.D., 500 frames counted.

FIGURE LEGENDS

Figure 1. Mouse testis, control unirradiated tissue. Note the numerous spermatogonia along the periphery of the seminiferous tubule (arrows), 1500x.

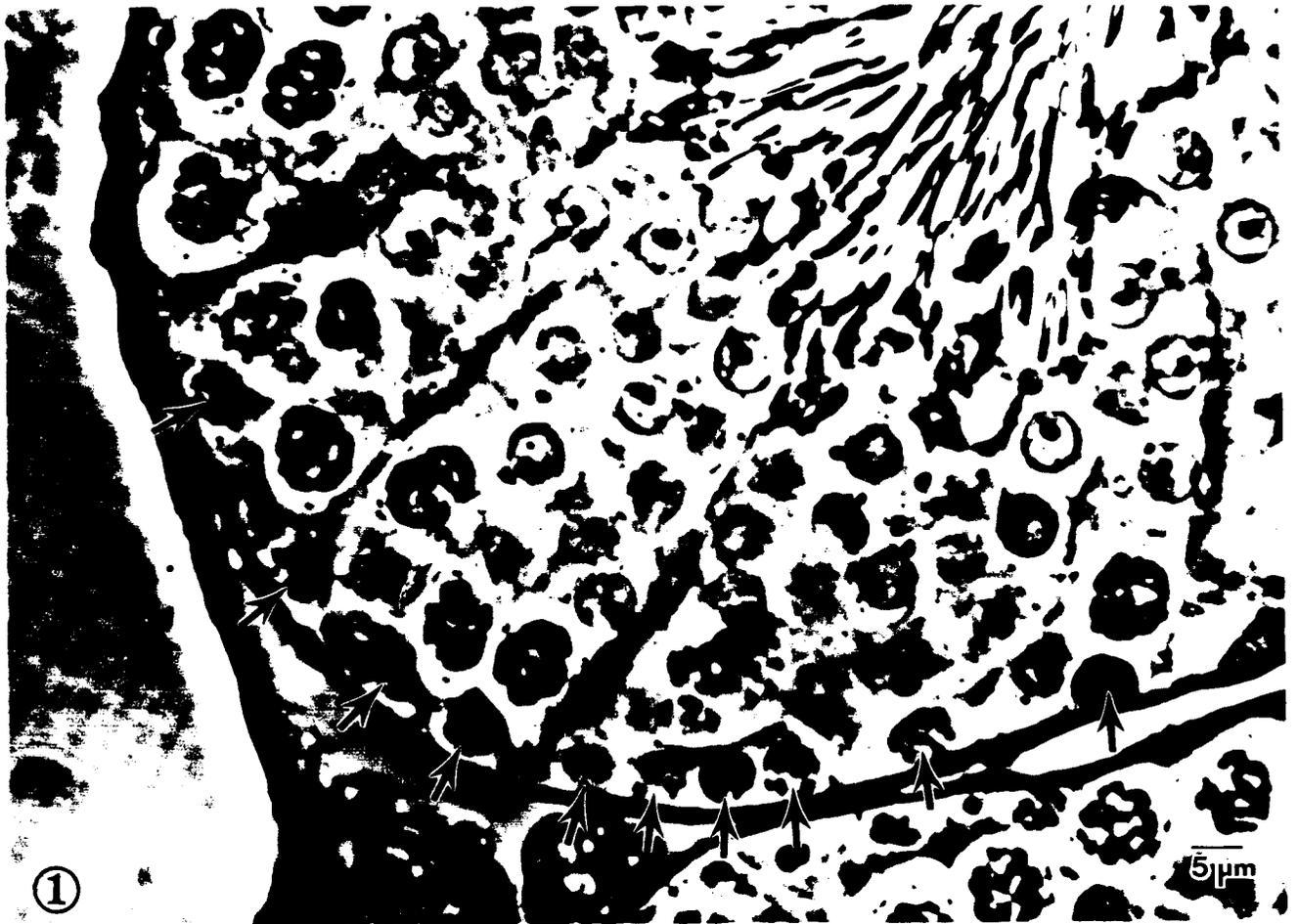
Figure 2. Mouse testis, 0.45 Gy Argon irradiation, 72 hours post-irradiation. Spermatogonia are absent. The periphery of the seminiferous tubule contains only spermatocytes (Spct) and Sertoli cells (Ser), 1500x.

Figure 3. Surviving fraction (S/S_0) of seminiferous epithelial cells after x-irradiation. Solid line indicates total number of spermatogonia in Stage 6 tubules; broken line indicates number of pre-leptotene spermatocytes in Stage 1 tubules.

Figure 4. Surviving fraction (S/S_0) of seminiferous epithelial cells after helium irradiation. Solid line indicates total number of spermatogonia in Stage 6 tubules; broken line indicates number of pre-leptotene spermatocytes in Stage 1 tubules.

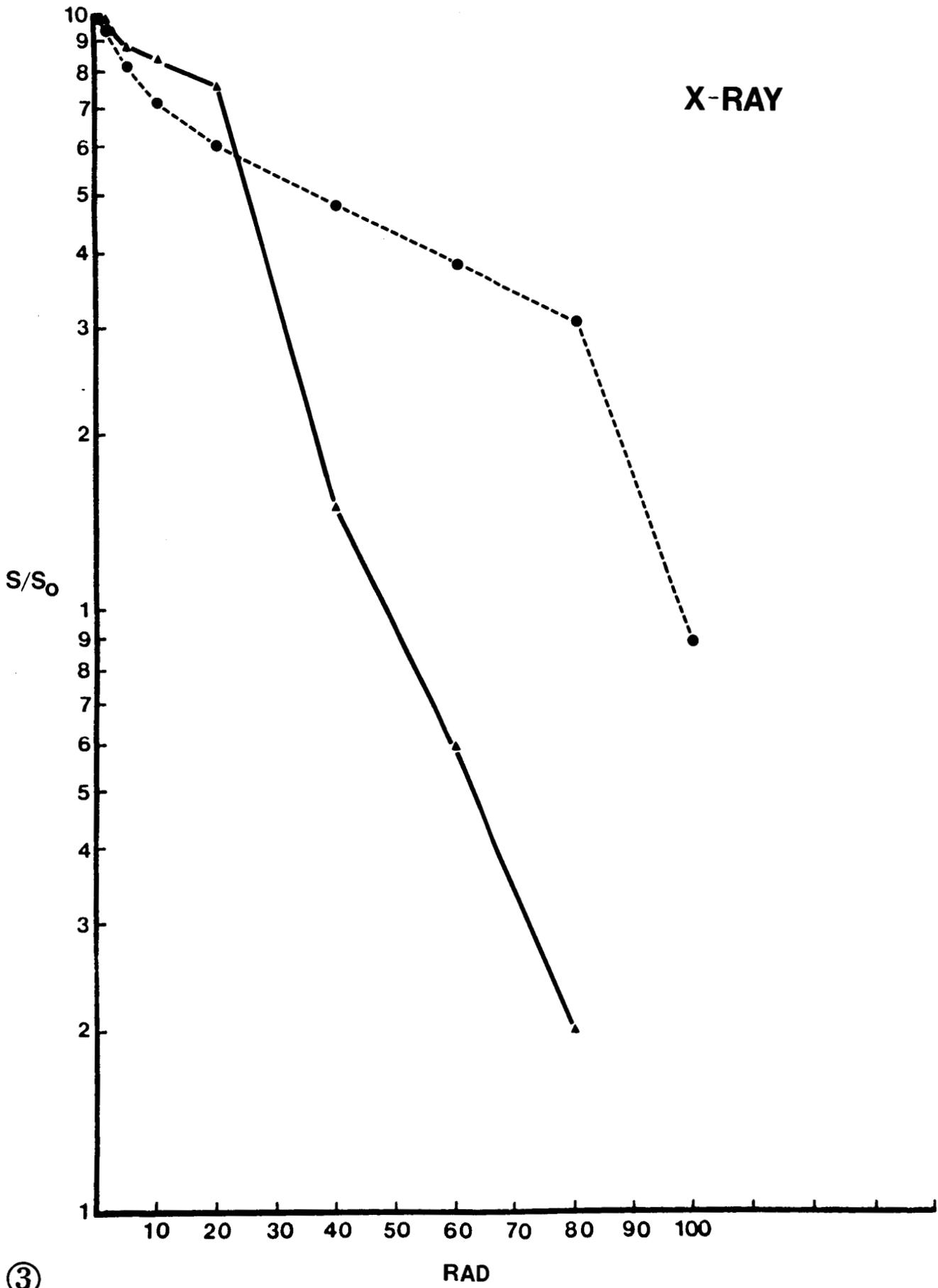
Figure 5. Surviving fraction (S/S_0) of seminiferous epithelial cells after Argon irradiation. Solid line indicates total number of spermatogonia in Stage 6 tubules; broken line indicates number of pre-leptotene spermatocytes in Stage 1 tubules.

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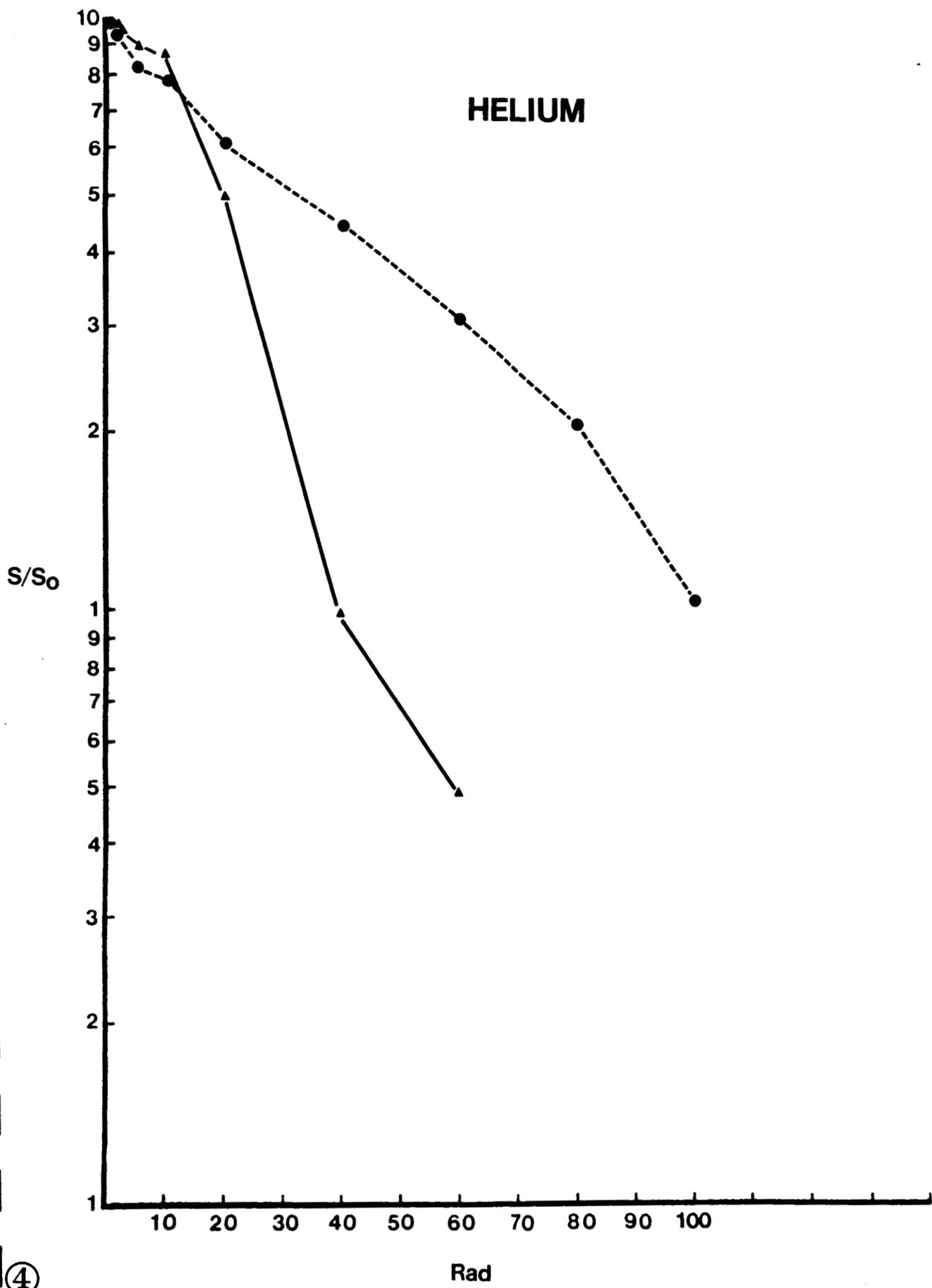
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HELIUM



ARGON

