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SERTOLI CELL DEATH BY APOPTOSIS IN THE IMMATURE RAT TESTIS FOLLOWING X-IRRADIATION

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Abstract

The importance of the morphological study of cell death has recently been emphasized by the recognition that the ultrastructural features of dying cells allow categorization of the death as either apoptosis or necrosis. This classification enables inferences to be drawn about the mechanism and biological significance of the death occurring in a particular set of circumstances.

In this study, Sertoli cell death induced in the immature testis of three and four day old rats by 5 Gy (500 rads) x-irradiation was described by light and transmission electron microscopy with the objective of categorizing the death as apoptosis or necrosis. The testes were examined 1, 2, 3, 4, 8, and 24 h after irradiation.

Following irradiation, there was a wave of apoptosis of the Sertoli cells starting in three to four hours and reaching a peak between four and eight hours. At 24 hours, only 61% of the expected number of Sertoli cells remained.

These findings are in accord with recent ultrastructural reports that ionizing radiation induces cell death by apoptosis in rapidly proliferating cell populations. New insights into the pathogenesis of radiation-induced cell death might thus be expected to stem from future elucidation of the general molecular events involved in triggering apoptosis.

Key Words: Cell death, cell survival, apoptosis, radiation, Sertoli cell, testis, immature, electron microscopy.

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Introduction

Radiation has long been known to cause transient infertility in the mature male by killing differentiating spermatogonia in the seminiferous epithelium (Oakberg, 1955), with Sertoli cell response playing a minor or negligible role (Kochar and Bateman, 1969; Erickson and Blend, 1976). In contrast, Sertoli cells in the immature testis may be a major element in determining the long-term irreparable loss of sperm-producing capacity induced by neonatal testicular irradiation (Erickson and Blend, 1976).

The radiosensitivity of Sertoli cells is correlated with their mitotic activity and state of differentiation (Harding, 1961; Courot, 1964; Erickson and Blend, 1976). In the adult they are fully differentiated, do not divide (Steinberger and Steinberger 1977) and are radioresistant (Fogg and Cowing, 1951; Kochar and Bateman, 1969; Erickson and Blend, 1976), whereas in young animals Sertoli cells are not fully differentiated, undergo mitosis and are radiosensitive (Harding, 1961). In the rat, Sertoli cells proliferate actively at and soon after birth with mitotic activity continuing during the first two weeks of postnatal life (Clermont and Perey, 1957; Steinberger and Steinberger, 1971; Ramos and Dym, 1979).

Radiation-induced spermatogonial death in the mature testis has recently been shown (Allan et al., 1987) to have the ultrastructural features that identify it as apoptosis (Wyllie et al., 1980), not classical necrosis. Precise morphological categorization of the Sertoli cell death following irradiation of the immature testis has, however, not been reported.

The value of using morphology to categorize the cell death occurring in a particular set of circumstances depends on the correlation that exists between the morphology of the death (either

necrosis or apoptosis) and its mechanism and biological significance. Necrosis is a passive degenerative phenomenon that inevitably follows irreversible disruption of a cell's homeostasis by injury. Morphologically, necrosis is characterized by swelling progressing to rupture of internal and plasma membranes and eventual total disintegration of organized cellular structure, usually associated with the development of inflammation. Apoptosis has features suggesting an active process of cellular self-destruction, and in many circumstances it is regulated by physiological stimuli (Kerr et al., 1972,1987; Wyllie et al., 1980; Wyllie, 1981; Searle et al., 1982; Cohen and Duke, 1984; Wyllie et al., 1984). Apoptosis involves rapid condensation of the nucleus and cytoplasm with preservation of organelles usually followed by nuclear and surface budding to produce membrane-bounded cell fragments (apoptotic bodies). Apoptotic bodies are subsequently phagocytosed intact and digested by surrounding resident cells (Kerr et al., 1972,1987; Wyllie et al., 1980; Wyllie, 1981).

In this paper, we present light and transmission electron microscopic evidence that death of immature Sertoli cells occurring in the first 24 h after x-irradiation takes the form of apoptosis.

Materials and Methods

Forty-eight male Sprague-Dawley rats, 37 aged 3 days (27 treated, 10 control) and 11 aged 4 days (8 treated, 3 control), were selected at random from a total of 12 litters. The animals were housed during the experiments in standard mouse boxes in a laboratory in which the ambient temperature was 26 ± 2°C. All treatments were performed between 0800 and 1000 hours.

Three animals at a time were restrained, unanesthetized, with cotton wool in a Petri dish and positioned so that the animals would be in the centre of the x-ray beam. Treated animals were exposed to 5 Gy (500 rads) x-irradiation using a Toshiba Therapy X-Ray Unit operated at 200 kV and 15 mA, with 2.0 mm Al filtration, 5.4 Gy/minute and target-testis distance of 8 cm. Dosages were measured using a Farmer dosimeter. Three day old irradiated animals were killed by decapitation at intervals of 1 (3 animals), 2 (6 animals), 3 (6 animals), 4 (6 animals), 8 (3 animals) and 24 (3 animals) hours after irradiation and the testes processed for transmission electron microscopy (TEM) and for light microscopy (LM). Four day old animals were similarly treated and killed 4 (3 animals), 8 (3 animals) and 24 (2 animals) hours after irradiation. Control animals were sham-irradiated and killed at the same times as the treated animals.

Transmission electron microscopy

One testis from each animal was placed in a solution of 3% glutaraldehyde in 0.067 M sodium cacodylate buffer, pH 7.2, for 24 h. The testes were then washed for 24 h in cacodylate buffer, postfixed in 1% osmium tetroxide for 2 h, washed in distilled water, stained in 5% aqueous uranyl acetate, dehydrated through a series of graded ethanol solutions, cleared in propylene oxide and embedded in an Epon/Araldite mixture.

Sections 1 µm in thickness were cut on an LKB Ultratome V and stained with toluidine blue for use in light microscopy, quantification and in selecting areas for electron microscopy. Ultrathin sections were picked up on uncoated copper grids, stained with lead citrate for 1-2 minutes and examined in an Hitachi H-300.

Light microscopy

The other testis from each animal was placed in Bouin's fixative for 24 h. After embedding in paraplast wax, 5 µm sections were cut and stained with haematoxylin and eosin. Toluidine blue stained, resin-embedded sections were also used for LM.

Quantification

Quantification was carried out on 3-day-old animals. One toluidine blue-stained section from three animals of each treatment group was used. Normal cellular components and cell death were quantified in 20 transverse circular profiles of seminiferous cords selected at random. The main interest was to establish the patterns of change following irradiation. No correction factors were applied to account for differences in cell size, change in cord profiles during normal growth, or for shrinkage of the cord profile with loss of irradiated (Sertoli) cells.

The following six categories were counted in each transverse profile: 1) Sertoli cell nuclei = S; 2) Sertoli cell mitoses = M; 3) Cells showing features of early apoptosis (characterized by condensation and margination of the nuclear chromatin in association with well preserved cytoplasm) = EA; 4) Cells showing features of late apoptosis (characteristic chromatin pattern recognizable but associated with degenerative changes in the cytoplasm and/or nucleus) = LA; 5) Degraded apoptotic bodies = D; 6) Gonocyte nuclei = G. In the case of normal Sertoli cells and gonocytes, only nuclei containing at least one nucleolus were counted. The counts were expressed as the mean number of each category per 20 tubular cross sections.

Statistical analysis

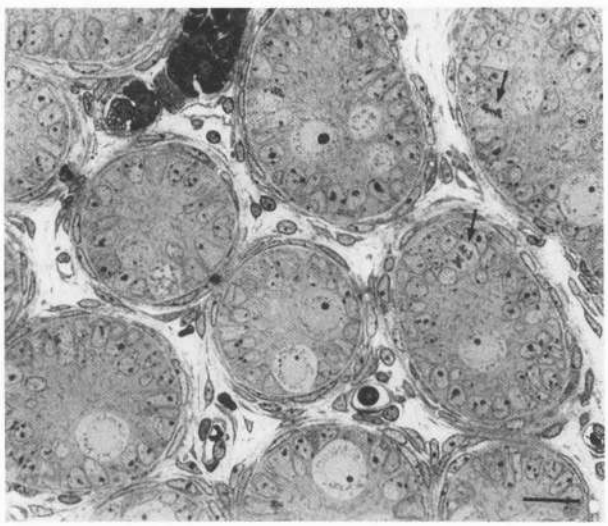
The change in the number of normal Sertoli cells and of early, late and degraded apoptotic bodies with time was examined by fitting quadratic regressions; counts of apoptotic bodies were very small or zero in many animals, so a square root transformation for counts was applied before fitting the regressions (Snedecor and Cochran, 1980).

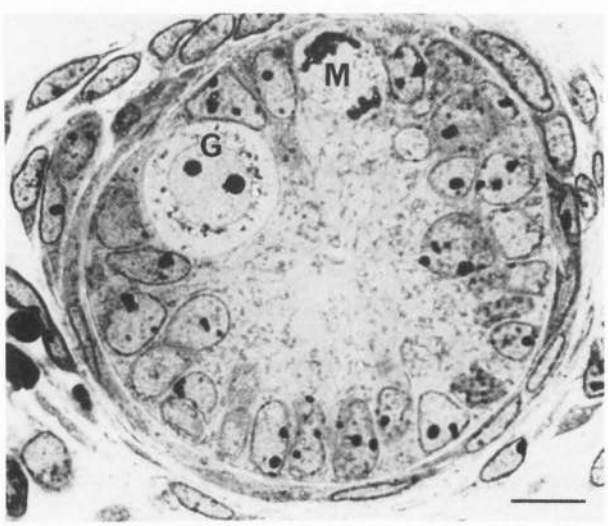
Results

Light Microscopy

The morphological appearances in the control animals were similar to those described by Franchi and Mandl (1964), Novi and Saba (1968) and Ramos and Dym (1979). The seminiferous tubule of the 3 day rat testis (Figs. 1 and 2) is represented by solid cords composed of only two cell types; the germ cells (gonocytes) located in the central zone of the tubules, and the supporting cells (Sertoli cells) lying adjacent to the basement membrane or intermingled among the gonocytes (Novi and Saba, 1968). Spontaneous cell death having the histological features of apoptosis was observed infrequently, approximately one apoptotic cell per 50 circular tubule profiles. Sertoli cell mitoses with "small" equatorial diameters of approximately 7 μm (Clermont and Perey, 1957) were prominent in the control animals (Figs. 1 and 2). Four day old testes were similar to the three day testes with, additionally, "large" mitotic figures of approximately 13 μm equatorial diameter indicative of gonocyte mitosis (Clermont and Perey, 1957) being present in some tubules.

The morphological appearances (LM and TEM) of the treated testes of 3 and 4





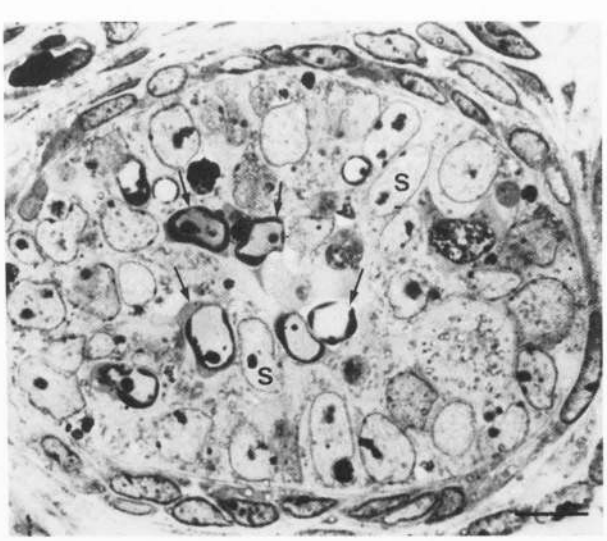


Figure 1. Control testis, 3 day animal. Several seminiferous tubules showing large, pale-staining gonocytes located in the central areas of most tubules. Sertoli cells, some with mitotic figures (arrows), are lying adjacent to the basement membrane. 1μm toluidine blue-stained section. Bar = 20 μm.

Figure 2. Control testis, 3 day animal. Higher power view of Sertoli cells, Sertoli cell mitosis (M) and a gonocyte (G). 1μm toluidine blue-stained section. Bar = 10 μm.

Figure 3. 4 h post-irradiation, 3 day animal. Early apoptotic bodies (arrows) derived from Sertoli cells and showing characteristic compacted, sharply-delineated chromatin marginated against the nuclear membrane. Sertoli cells at S and elsewhere. 1μm toluidine blue-stained section. Bar = 10 μm.

day old animals were similar and will not be described separately.

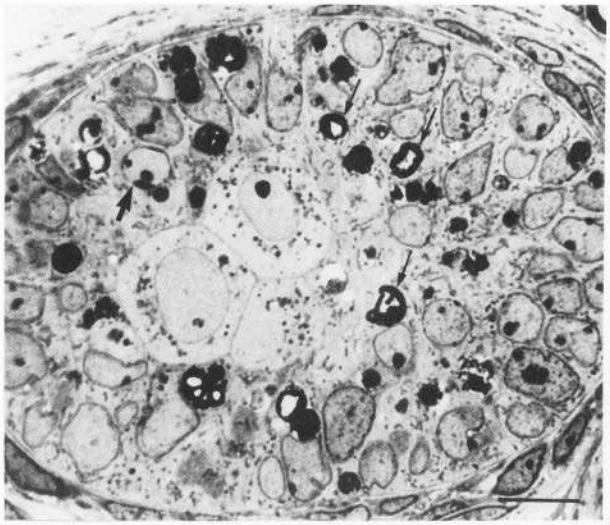
Histological evidence of increased apoptosis was apparent by 3 h after irradiation and very prominent at 4, 8 and 24 h (Figs. 3, 4, and 5).

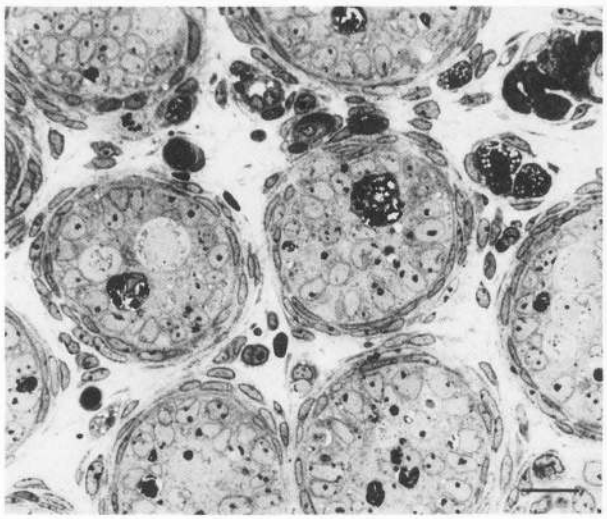
The early apoptotic bodies, which were evident from 3 h onwards, were round, ovoid, or less frequently irregular in shape, showed increased cytoplasmic staining and contained either a nucleus with marginated, condensed chromatin in sharply-delineated, crescentic configurations or a uniformly pyknotic nuclear chromatin mass (Fig. 3). Late apoptotic bodies, showing some nuclear changes characteristic of apoptosis but having intensely stained, featureless cytoplasm (Fig. 4) were common after 4 h. Dense masses of cell debris (Figs. 3 and 4) retaining no identifiable characteristics of apoptosis became more prominent with time after irradiation. By 24 h, the evidence of cell death was most obvious as dense, relatively amorphous cell remnants (Fig. 5) which, in some tubules, were aggregated within the cytoplasm of a single phagocytic cell (Fig. 6). Cells showing the features of early apoptosis were present in only small numbers at this time.

Gonocytes were not observed to die following irradiation.

Transmission electron microscopy

Electron microscopy confirmed the marked condensation of nuclear chromatin in early apoptotic bodies that was observed by light microscopy and the frequent location of the condensed chromatin in discrete peripheral masses





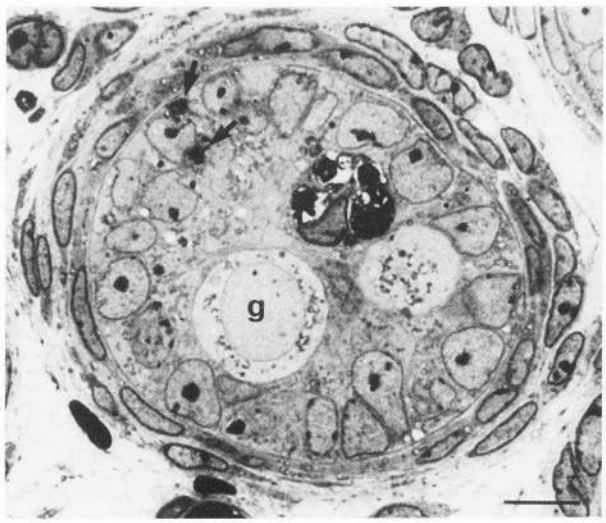
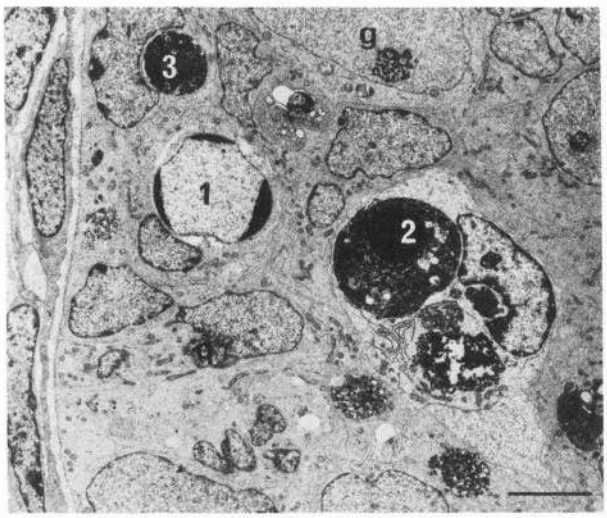
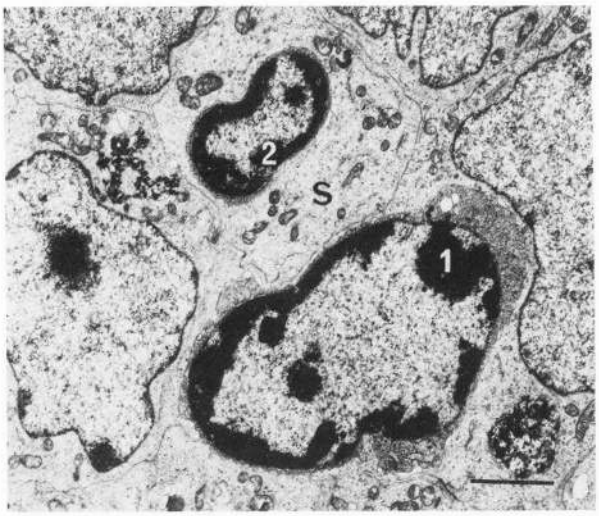


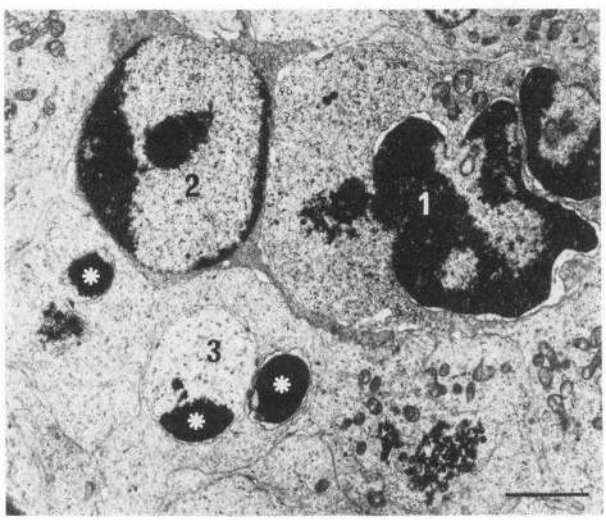
Figure 4. 8 h post-irradiation, 3 day animal. Early apoptosis (thick arrow). Late apoptotic bodies (arrows) retain evidence of marginated chromatin. The other dense masses of cell debris are most probably phagocytosed apoptotic bodies in advanced stages of degradation. 1µm toluidine blue-stained section. Bar = 10 µm.

Figure 5. 24 h post-irradiation, 3 day animal. Cell debris in advanced stages of degradation either scattered throughout the tubule or aggregated into large masses. 1µm toluidine blue-stained section. Bar = 20 µm.

Figure 6. 24 h post-irradiation, 3 day animal. Degraded cell debris accumulated within a single phagocyte or evident as small masses (arrows) within the cytoplasm of a peripheral Sertoli cell. g = gonocyte. 1µm toluidine blue-stained section. Bar = 10 µm.







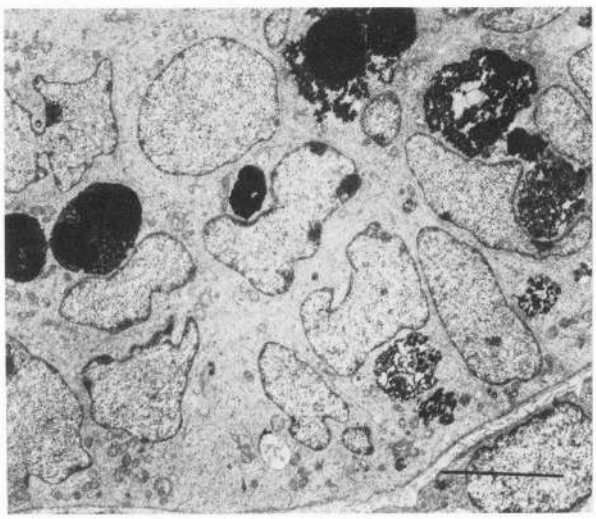


Figure 7. Electron micrograph, 4 day animal, 4 h after irradiation. An early apoptotic body (1) shows the characteristic masses of condensed, peripherally located nuclear chromatin. Advanced degenerative changes following phagocytosis are evident in 2 and 3. g = gonocyte. Bar = 5 μm.

Figure 8. 4 h post-irradiation, 4 day animal. Sertoli cells (1,2) showing the early nuclear changes of apoptosis with condensed, marginated chromatin. The nucleus of Sertoli cell 3 has 'budded' into smaller membrane-bounded fragments (*). Bar = 2 μm.

Figure 9. 4 h post-irradiation, 4 day animal. Two early apoptotic bodies (1, 2) with marginated nuclear chromatin. 1 has a thin rim of condensed cytoplasm. 2 is also almost entirely nuclear in composition and is surrounded by cytoplasm of an engulfing Sertoli cell (S). Bar = 2 μm.

Figure 10. 8 h post-irradiation, 4 day animal. Numerous apoptotic bodies in various stages of degradation within phagolysosomes of viable resident Sertoli cells. Bar = 5 μm.

(Figs. 7, 8 and 9). Fragmentation of the nucleus and cytoplasm produced smaller membrane-bound apoptotic bodies (Figs. 8 and 9). Fragmentation of the nucleus, as distinct from nuclear folding, was confirmed by the ultrastructural finding of isolated nuclear remnants within small, phagocytosed apoptotic bodies (Fig.9). The cytoplasm of apoptotic bodies was condensed (Figs. 8 and 9) and, in most cases, the organelles were well preserved (Fig. 8). The small size of the mitochondria in early apoptotic

bodies (Fig. 8) confirmed their origin from Sertoli cells; the mitochondria of gonocytes are several times larger in average dimensions (Fig. 7). There was no evidence that apoptotic bodies were derived from gonocytes following irradiation.

In other apoptotic bodies, there was progressive organelle degeneration beginning with mitochondrial swelling. Where apoptosis has been studied in other tissues, such degenerative changes have usually been observed to postdate phagocytosis of the bodies (Wyllie et al., 1980; Searle et al., 1982). Advanced degeneration, presumably occurring within Sertoli cell phagolysosomes, was associated with complete loss of recognizable cytoplasmic components (Figs. 7 and 10). Masses of condensed chromatin, characteristic of apoptosis, could however often still be identified within such degraded bodies (Fig. 10).

The partly degraded remnants of apoptotic bodies (Figs. 7 and 10) showed ultrastructural changes that bear a resemblance to cellular necrosis as seen in other tissues (Trump and Mergner, 1974). Nevertheless, no positive evidence for necrotic Sertoli cells was detected. At short intervals following radiation, relatively well preserved apoptotic bodies were prominent. No cells showing both nuclear and cytoplasmic changes of early necrosis (Trump and Ginn, 1969; Reimer et al., 1972; Trump et al., 1973) were found. At later intervals, partly degraded cellular remnants were observed with increasing frequency, consistent with the occurrence of a wave of apoptosis.

An unusual feature found at late times after irradiation was the phagocytosis of apoptotic bodies derived from Sertoli cells by a small proportion of gonocytes (Figs. 11 and 12).

Quantification

The quantitative effects of irradiation on the 3 day testis are shown in Tables 1 and 2, and in Figure 13. The number of Sertoli cells declined with increasing time after irradiation; at 24h 61% of the expected number were present (Table 1). The square root of Sertoli cell number showed a significant linear decline with time and a significant curvilinear relationship with time, the values decreasing more rapidly at earlier times (Table 2). Mitosis of Sertoli cells was inhibited at all times after irradiation whereas death of Sertoli cells increased from 3 h after irradiation.

Early apoptosis was enhanced at 3 h and prominent at 4 h with late apoptosis and degraded apoptotic bodies predominating at later times (Table 1, Figure 13). The square root of the number of early, late and degraded apoptotic bodies showed significant linear declines with time and significant curvilinear relationships with time; in each case the curve showed a rise to maximum value followed by a decline (Table 2).

The number of gonocytes was not affected by irradiation (Table 1).

Table 1. Radiation effects on 3 day old rat testis

	Mean number per 20 tubule profiles					
Group	S*	M	EA	LA	D	G
Control						
1-8h (5)**	417	14	0.4	0.6	0.2	17
24 h (3)	416	14	0.3	0	0.3	18
Radiation						
1 h (3)	401	0	0	0	1.7	17
2 h (3)	349	0	0.6	0	0.3	18
3 h (3)	368	0	6.0	5.3	6.3	17
4 h (3)	341	0	35.7	30.7	38.7	16
8 h (3)	291	0	4.7	35.3	85.3	20
24 h (3)	254	3	4.7	16.3	60.7	21

* S = Sertoli cell nuclei. M = Sertoli cell mitosis. EA = early apoptosis. LA = late apoptosis. D = degraded apoptotic bodies. G = gonocyte nuclei.

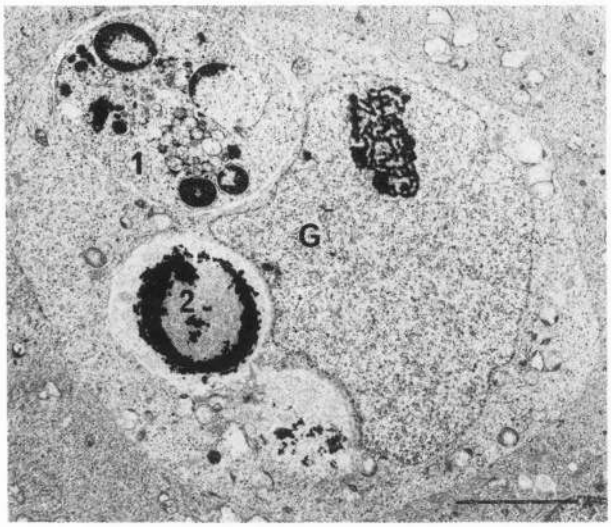
** the number of animals in the group used to derive the mean values

Table 2. Regression of square root of cell number on time

	Regression Coefficients	
	linear	quadratic
Sertoli cells	-0.529**	0.014**
early A B's	0.617**	-0.023*
late A B's	1.137**	-0.040**
degraded A B's	1.586**	-0.054**

(A B's = apoptotic bodies)

* significant at 5% level
** significant at 1% level



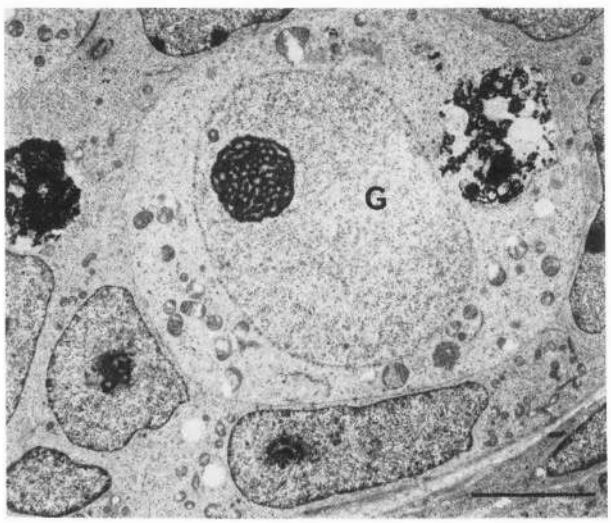


Figure 11. 24 h post-irradiation, 3 day animal. Gonocyte (G) with prominent nucleolus and pale staining cytoplasm contains phagocytosed apoptotic bodies (1 and 2) derived from Sertoli cells. Note the small nuclear fragments in 1 and nuclear changes characteristic of apoptosis in 1 and 2. Bar = 5 μm.

Figure 12. 24 h post-irradiation, 3 day animal. Phagocytosed apoptotic body in advanced stage of degradation within the cytoplasm of a gonocyte (G). The gonocytes are easily identified as they are bigger than the Sertoli cells, their cytoplasm stains more lightly, they have an extremely prominent nucleolus, and their mitochondria are significantly larger. Bar = 5 μm.

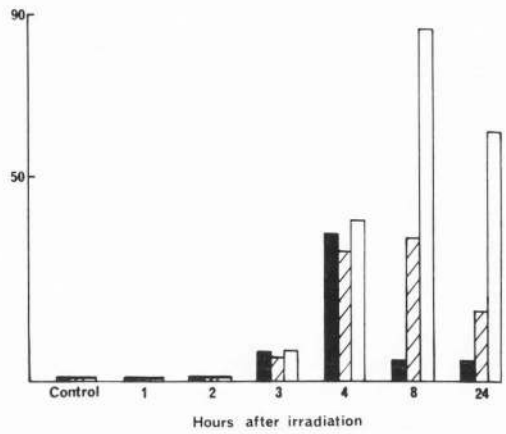


Figure 13. Radiation-induced Sertoli cell death in the seminiferous epithelium of the 3 day old rat. The mean number of counts per 20 cross sections is plotted against time following 5 Gy x-irradiation.
■ = early apoptosis.
▨ = late apoptosis.
□ = degraded apoptotic bodies.

Discussion

Our electron microscopic findings establish that the death of immature Sertoli cells that follows x-irradiation takes the form of apoptosis. This is in accord with recent ultrastructural observations of acute radiation-induced cell death occurring in a variety of other tissues (Kerr and Searle, 1980; Allan et al., 1987; Harmon and Allan, 1988). Past descriptions of Sertoli cell death in the irradiated immature testis have mostly referred to it by non-specific terms such as degeneration or pyknosis (Harding, 1961). There have been few electron microscopic studies, and the death has not previously been precisely categorized morphologically. Harding (1961) has previously reported the inhibition of Sertoli mitosis following irradiation of neonatal rat testes.

Gonocytes, the primordial germ cells present in the neonatal testis, did not show evidence of radiation-induced death in this study. The morphological changes in gonocytes are known to be delayed for some time after irradiation, usually occurring at approximately the time when the gonocytes undergo mitosis to produce spermatogonia for the initiation of spermatogenesis (Courot, 1964; Erickson, 1963; Franchi and Mandl, 1966). In the rat, gonocyte mitosis occurs at about the fourth or fifth postnatal day (Novi

and Saba 1968). Quantification of cell death in the irradiated 3 day testis in our study reflects death of Sertoli cells only and avoids the difficulty of identifying the origin of dying cells when older animals are used (Harding, 1961).

The present quantitative results are consistent with the x-rays having induced a wave of apoptosis in Sertoli cells beginning 3 to 4 h after exposure and rising to a peak between 4 and 8 h. The speed with which morphological changes associated with early apoptosis occur followed by the more prolonged late changes and ultimate degradation of the apoptotic bodies are characteristic features of apoptosis as described in a variety of other tissues (Wyllie et al., 1980; Searle et al., 1982; Kerr et al., 1984). Apoptotic bodies are often small and may be rather inconspicuous. They are rapidly disposed of by adjoining cells. The ultrastructurally diagnostic early stages of the process are extremely transient. Timing of observations after irradiation is critical for both categorization and direct quantification of the death produced.

Early apoptotic bodies are particularly useful for quantification of radiation-induced cell death as the type of cell death and the cells of origin are unmistakable at this stage. Later when the cell has 'budded' into a cluster of apoptotic bodies, or when the bodies have been phagocytosed and degraded within phagolysosomes, the distinction and accurate quantification is more difficult. Recognition of the time course of the sequence of morphological changes during the production and degradation of apoptotic bodies is essential.

The significance of apoptosis, and not necrosis, being the type of cell death induced by x-irradiation is uncertain. There is evidence that apoptosis is an active process of cellular self-destruction (Wyllie et al., 1980), which has been shown, at least in some circumstances, to require RNA and protein synthesis for its execution (Cohen and Duke, 1984; Wyllie et al., 1984).

The way in which apoptosis is triggered is still poorly understood. It has been suggested that certain rapidly proliferating cell populations possess intrinsic mechanisms for the controlled, selective elimination of cells with critical DNA damage via apoptosis, thus minimizing the propagation of significant genetic errors (Searle et al., 1975; Kerr and Searle, 1980; Wyllie et al., 1980). In the normal and x-irradiated mature testis (Allan et al., 1987), synchronous deletion of groups of linked spermatogonia by apoptosis might be consequence of passage of a newly synthesized effector substance from cell-to-cell via intercellular bridges. Other investigators have provided both morphological (Huckins and Oakberg, 1971; Huckins, 1978) and kinetic (Oakberg and Clark, 1961) evidence that radiation-induced damage to a single member of a spermatogonial chain results in death of all members of the chain.

New insights into the pathogenesis of radiation-induced cell death might be expected to stem from future elucidation of the general molecular events involved in triggering apoptosis under both physiological and pathological circumstances.

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Discussion with Reviewers

H. Grier: What similarities are there between apoptosis as a process and the normal process of residual body formation during spermiogenesis?
Authors: Both processes result in the formation of membrane-bound cell fragments which are phagocytosed by neighboring resident cells. In apoptosis, enzymatic cleavage of double-strand DNA and nuclear fragmentation are characteristic features associated with death of the cell; apoptotic bodies contain cytoplasmic organelles of all types often in association with nuclear remnants. Residual bodies on the other hand contain selected cytoplasmic organelles, mitochondria are excluded, and compacted, single-strand DNA is preserved intact in the mature spermatid.
H. Grier: What might the cellular signals be for ingestion of residual bodies by Sertoli cells or the ingestion of apoptotic bodies by surrounding resident cells?
Authors: The avidity with which apoptotic bodies are taken up by adjacent cells probably depends on changes in the nature of the carbohydrates exposed on their surface (Duval E, Wyllie AH, Morris RG. 1985. Macrophage recognition of cells undergoing programmed cell death [apoptosis] . Immunology 56, 351-358).