

Identification of male germ cells undergoing apoptosis in adult rats

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The possible role of apoptosis in spontaneous or induced germ cell death was investigated by treating adult male rats with either a GnRH antagonist ($112.5 \mu\text{g kg}^{-1} \text{day}^{-1}$ for 14 days) or methoxyacetic acid ($650 \mu\text{g kg}^{-1}$; single dose) or sham-treated with either of the vehicles ($n = 3$ per group). The antagonist virtually abolished gonadotrophin secretion, while methoxyacetic acid reduced serum testosterone concentrations and slightly increased those of FSH (neither significantly). Bands of low molecular mass characteristic of apoptotically degraded DNA were detected by electrophoresis in both treatment groups but not in the controls. Sectioned, Carnoy-fixed testes were screened for degenerating cells with periodic acid–Schiff's base and haemalaun or examined for apoptotic cells using a modified *in situ* end-labelling procedure. Periodic acid–Schiff's-stained dying cells were found in low numbers in control animals with a distribution and frequency that matched that of apoptotic cells. Degenerating germ cells identified by histology were present at certain stages of spermatogenesis after 2 weeks of antagonist treatment. A comparison of their distribution with that of end-labelled cells identified the cell death as apoptotic. Methoxyacetic acid caused a massive depletion of spermatocytes at stages IX–II, which was also found to be apoptotic. It is concluded that spontaneous germ cell death in adult rats is apoptotic and that both gonadotrophin ablation and administration of methoxyacetic acid can cause apoptosis in the germ cells of adult male rats, but via different routes.

Introduction

Apoptosis is a process of physiological cell death characterized by certain morphological (cytoplasmic and nuclear condensation, membrane blebbing) and biochemical (inter-nucleosomal DNA cleavage) characteristics (Kerr *et al.*, 1972; Wyllie, 1980, 1987; Wyllie *et al.*, 1984). It is induced by specific cues that initiate a gene-directed, endogenous, cell-death programme that in turn drives DNA cleavage, cytoplasmic and nuclear condensation and fragmentation, and changes the characteristics of the plasma membrane to promote phagocytosis by surrounding cells (Williams and Smith, 1993). Apoptosis has been observed in a wide variety of tissues and species, where it occurs in a range of processes such as embryonic development, lymphocyte maturation, tumour regression and as a facultative response to genetic damage (for a review see Schwartzman and Cidlowski, 1993).

Spontaneous death of germ cells is a widespread, but little-understood, phenomenon in the testes of many species (Roosen-Runge, 1973; Huckins, 1978; Allan *et al.*, 1987). In mammals, it is common in all species studied, including rats (Wing and Christensen, 1982), and primates (Johnson *et al.*, 1987, 1990; Russell *et al.*, 1990). The reason for this wastage is unknown, but an understanding of the mechanisms involved could be useful in the understanding of male infertility and in the development of methods of male contraception. It was

therefore decided to investigate whether spontaneous cell death during spermatogenesis is apoptotic, as this would indicate the presence of certain molecular events that might themselves be amenable to further study and possible manipulation.

It has been shown that testicular cell loss induced by hypophysectomy in immature rats is correlated with the appearance of an apoptosis-specific marker (Tapanainen *et al.*, 1993), namely a characteristic 'ladder' pattern seen after electrophoresis of the DNA (Wyllie *et al.*, 1984). Apoptosis results in the cleavage of DNA at internucleosomal regions leading to fragments of approximately 185 base pairs, or multiples of this size (Wyllie, 1980) that separate during electrophoresis to give the diagnostic ladder. After hypophysectomy, this effect was shown to be present in the interstitial and seminiferous tubule compartments, but the specific cell types undergoing apoptosis could not be identified.

In situ end-labelling methods for the localization of apoptotic cells in somatic tissues were reported by Gavrieli *et al.* (1992) and Wijsman *et al.* (1993). These are based on the same principle: the detection of biotinylated nucleotides incorporated at the 3' ends of DNA strands. Since inter-nucleosomal cleavage of DNA is a characteristic feature of apoptosis in most cell types and results in the incorporation of many more nucleotides than in unaffected cells; those undergoing apoptosis can be distinguished by standard biotin-detection systems.

The first objective of this study was to develop a method for identifying apoptotic cells in the testes; the second was to

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investigate the occurrence of apoptosis among the different germ cell types. The method developed was tested by using a GnRH antagonist, a treatment that had already been found to cause apoptosis in the testes of immature rats (Tapanainen *et al.*, 1993). Methoxyacetic acid was used to explore further the induction of apoptosis in the testis. This compound is highly toxic to rat pachytene spermatocytes (Anderson *et al.*, 1987) across all stages of spermatogenesis (Foster *et al.*, 1983, 1984; Creasy *et al.*, 1985). Whether this death is apoptotic or necrotic has not been investigated but it is known that concentrations of gonadotrophins are only slightly altered after treatment with methoxyacetic acid (Bartlett *et al.*, 1988; Allenby *et al.*, 1991) suggesting that hormone imbalances are not the principal cause.

Materials and Methods

Experimental design

Twelve adult male Sprague–Dawley rats (Charles River Deutschland, Sulzfeld), weighing approximately 400 g and housed under standard conditions, were evenly distributed among four groups. Animals in each group were weighed every third day and treated as follows: Group 1 – 0.9% (w/v) saline, 0.5 ml kg⁻¹, s.c., daily for 14 days; Group 2 – 112.5 µg GnRH antagonist kg⁻¹ (Cetrorelix, ASTA Medica, Frankfurt) in 0.9% saline, 0.5 ml kg⁻¹, s.c., daily for 14 days; Group 3 – 0.9% saline pH 7.4, 5 ml kg⁻¹, single i.p. dose; and Group 4 – 650 mg methoxyacetic acid kg⁻¹ (Aldrich-Chemie, Steinheim) (NaOH-buffered to pH 7.4) in 0.9% saline, 5 ml kg⁻¹, single i.p. dose.

Doses were chosen because they are known to cause high numbers of germ cells to die but still allow the seminiferous tubule stages to be identified (GnRH antagonist: G. Rosiepen, personal communication; methoxyacetic acid: Foster *et al.*, 1983, 1984; Creasy *et al.*, 1985; Sharpe *et al.*, 1993).

Groups 3 and 4 were treated on the day that groups 1 and 2 received their final dose. Twenty-four hours later, all animals were weighed and then killed by decapitation after mild anaesthesia with CO₂. Trunk blood was collected into tubes and allowed to coagulate overnight at 4°C for the subsequent removal of serum, which was then stored at -20°C until required. The testes were removed, weighed and portions fixed either in Carnoy's fluid, Bouin's fluid or 4% (w/v) buffered formalin in PBS, or, frozen rapidly in liquid N₂ and stored at -80°C. Fixed tissues were processed by conventional methods and embedded in paraffin wax.

Hormone assays

Sera were analysed for concentrations of testosterone and FSH as described by Chandolia *et al.* (1991a). Each hormone was analysed in a single run. Testosterone was assayed by double-antibody radioimmunoassay; the detection limit was 0.7 nmol l⁻¹ and the intra-assay coefficient of variation was 6.2%. For the FSH radioimmunoassay, FSH-RP-2 was used as the standard, FSH-I-6 as tracer and anti-rFSH-S-11 as anti-serum. The detection limit and coefficient of variation were 0.8 ng FSH-RP-2 ml⁻¹ and 9.4%, respectively.

Histochemistry and histology

Sections (2 µm) from fixed testes were deparaffinized, rehydrated, rinsed in terminal-deoxytransferase (TdT) buffer (Gavrieli *et al.*, 1992) and incubated for 1 h with 10.0 µmol biotinylated deoxyuridine triphosphate l⁻¹ (bio-16-dUTP) (Boehringer Mannheim GmbH, Mannheim) and 0.03 U TdT µl⁻¹ (Promega, Madison, Serva Feinbiochemica, Heidelberg), in the same buffer. The concentration of bio-16-dUTP used was based on Wijsman *et al.* (1993). The reaction was ended by rinsing the slides thoroughly in Tris-buffered saline (TBS) and the biotinylation visualized with Extravidin–peroxidase complex (Sigma Chemie, Deisenhof) and diamino-benzidine, using conventional procedures. Slides were counterstained by brief immersion in Mayer's haemalaun solution (Merck, Darmstadt), rinsed in deionized water, washed thoroughly in running tap water, dehydrated and mounted. Comparable sections from each sample used for end-labelling were mounted on slides using 70% (v/v) alcohol, deparaffinized, rehydrated, stained by the periodic acid–Schiff's (PAS) method (15 min 1% (w/v) periodic acid, 15 min running tap water, 5 min deionized water, 45 min Schiff's reagent (Schmid, Köngen), 15 min running tap water, 5 min deionized water), counterstained with Mayer's haemalaun (5 min), differentiated with a brief rinse in aqueous 0.1% (v/v) HCl, washed in running tap water until clear staining of the elongating spermatid acrosomes was achieved, then dehydrated and mounted.

DNA ladders

Tissue frozen at -80°C provided material for DNA extraction by cell lysis and column purification using Quiagen-tips (Quiagen GmbH, Hilden) according to the manufacturer's instructions. Four µg DNA per animal or 1 µg of a 100 base pair DNA ladder (Pharmacia, Freiburg) as a marker was end-labelled in 100 mmol cacodylate buffer l⁻¹ pH 6.8, 26 mmol CoCl₂ l⁻¹, 0.1 mmol dithiothreitol l⁻¹, 0.1 mg BSA ml⁻¹, containing 50 µmol digoxigenin-16-dideoxyuridine ml⁻¹ (Boehringer Mannheim GmbH) and 0.03 U TdT µl⁻¹, for 1 h at 37°C. The DNA was precipitated by the addition of 0.1 volume 3 mol sodium acetate l⁻¹ and 2 volumes ethanol, washed twice in 100% (v/v) ethanol and once in 70% (v/v) ethanol, and then loaded onto a 2% (w/v) agarose gel (1% agarose (Serva Feinbiochemica); 1% (w/v) NuSieve agarose (Biozym Diagnostik, Hess. Oidendorf)) in TBE buffer (Sambrook *et al.*, 1989) and subjected to electrophoresis for 1 h at 140 V (10 V cm⁻¹). After Southern blotting onto Hybond-N nylon membrane (Amersham Buchler GmbH and Co. KG, Braunschweig), the DNA was fixed by UV light and the membrane incubated in blocking reagent (Boehringer Mannheim GmbH), for 30 min and washed 3 times for 10 min in TBS. It was then incubated for 1 h in TBS containing anti-digoxigenin–alkaline phosphatase complex (Boehringer Mannheim GmbH) diluted 1:1000, washed as before in TBS, rinsed in TBS pH 9.5 containing 50 mmol MgCl₂ l⁻¹, and then incubated for 5 min in the same buffer containing Lumigen PPD (Boehringer Mannheim GmbH) diluted 1:100. Finally, the blot was covered in plastic wrap, incubated for 15 min at 37°C, and then exposed to X-ray film (Hyperfilm-MP, Amersham Buchler GmbH and Co. KG), for 2–15 min.

Table 1. The effect of treatment with a GnRH antagonist or methoxyacetic acid on various reproductive parameters in male rats

Group	Mass of testes (g)	FSH (ng ml ⁻¹)	Testosterone (nmol l ⁻¹)	Degenerating cells ^a	
				PAS-stained	End-labelled
ANT control	3.83 ± 0.056	8.90 ± 1.358	18.93 ± 4.268	0.02 ± 0.000	0.03 ± 0.000
ANT treated	2.51 ± 0.109***	ND	ND	7.90 ± 0.361***	7.47 ± 0.520***
MAA control	3.99 ± 0.088	7.83 ± 2.048	9.20 ± 2.892	N/A	N/A
MAA treated	3.70 ± 0.104	11.43 ± 1.033	2.53 ± 0.260	N/A	N/A

ANT: GnRH antagonist; MAA: methoxyacetic acid; PAS: periodic acid-Schiff's. Values are means ± SEM. Statistical comparisons (Student's *t* test) were made between control and respective treatment group (*n* = 3 in all cases). ****P* ≤ 0.001. ^aValues are means per cross-section and were compared (Student's *t* test) within groups. ND: not detectable. N/A: it was impossible to quantify these cells accurately. These findings are therefore described in the text and illustrated in Fig. 3e–f.

Microscope analyses

Stages were classified according to the criteria described by Leblond and Clermont (1952) and by Hess (1990). Three testicular cross-sections per animal were examined for the presence of biotinylated and PAS-positive cells in all of the 14 germ cell associations in all four groups. Tubules were examined under the microscope with up to ×630 total magnification.

Degenerating cells in 25 stage VII cross-sections per animal in groups 1 and 2 were scored in the end-labelled and the PAS-stained sections. Stage VII was chosen as it has the lowest incidence of spontaneously degenerating cells (Kerr, 1992) but is the most sensitive to the effects of gonadotrophin withdrawal (Russell and Clermont, 1977; Cameron and Muffly, 1991; Chandolia *et al.*, 1991b; Kerr *et al.*, 1992).

In rats, the spermatogenic stages IX–XIV are particularly strongly affected by a single dose of 650 mg methoxyacetic acid kg⁻¹ (Foster *et al.*, 1983, 1984; Creasy *et al.*, 1985; Sharpe *et al.*, 1993). Quantification of these results proved to be inappropriate because at the late stages, in which considerable degradation was apparent, it was often difficult to distinguish apoptotic cells from apoptotic bodies produced following the fragmentation of a cell.

Statistical analyses

Data were analysed by two-sample *t* tests using computer software. The level of significance was set at 5%.

Results

The effect of the treatments on various reproductive parameters is shown (Table 1). The masses of testes were reduced by both treatments. However, the reduction was statistically significant only for the treatment with the GnRH antagonist. Serum gonadotrophin concentrations were markedly reduced in the animals treated with the GnRH antagonist to values not detectable by the assays used. Methoxyacetic acid had no significant effect on the hormones, although serum FSH was slightly higher and serum testosterone slightly lower than the concurrent control values.

There was no apparent difference between treatment groups and their respective controls in terms of the effect on body

mass, indicating that the treatments are unlikely to have significantly affected the health of the animals (data not shown).

Apoptosis in the testes of all animals of both treatment groups was demonstrated by the presence of bands of low molecular mass DNA, that were absent from the control groups. Bands of approximately 90, 185 and 370 are visible (Fig. 1); additional bands, of approximately 555 and 770, were visible on longer exposure (not shown). The intensity of the low molecular mass bands was much greater in the group treated with methoxyacetic acid than in the group treated with GnRH antagonist.

Degenerating cells were detected by both PAS staining and end-labelling, with considerable variation in their distribution (Fig. 2). Only the Carnoy-fixed tissues were found to be suitable for histological analysis and DNA end-labelling. Therefore, only these will be considered further. In control animals, dying cells were rarely observed and those that were present were at stages VII–I and were homogeneously stained blue to pink; a chromatin pattern was rarely seen. On the basis of their position within the epithelium, their size and the stages in which they were located, such cells were identified as A spermatogonia, leptotene, zygotene and early pachytene spermatocytes; dividing secondary spermatocytes in stage XIV were occasionally seen, and very rarely, a step-7 spermatid at stage VII. An almost identical result was obtained for the distribution and location of end-labelled cells, except that no labelled cells were found in stages VII or VIII. There was no discernible difference between the two control groups in terms of the incidence of dying cells. The distribution of degenerating cells seen by each method in the control groups is summarized (Fig. 2a).

Administration of the GnRH antagonist for 14 days visibly reduced the diameter of the seminiferous tubules and the number of elongating and elongated spermatids. Germ cells positive for PAS were distributed throughout stages VII–XIV. Occasionally, such cells were also visible at early stages, but because the treatment had totally depleted the elongating spermatids in this part of the cycle, reliable identification of the stages was not possible. Characteristically the degenerating cells were homogeneously stained, although in some cells condensed chromatin was visible, and these cells were frequently larger than normal. Biotin-labelled cells (Fig. 3a) had the same distribution pattern as that of PAS-positive cells. This

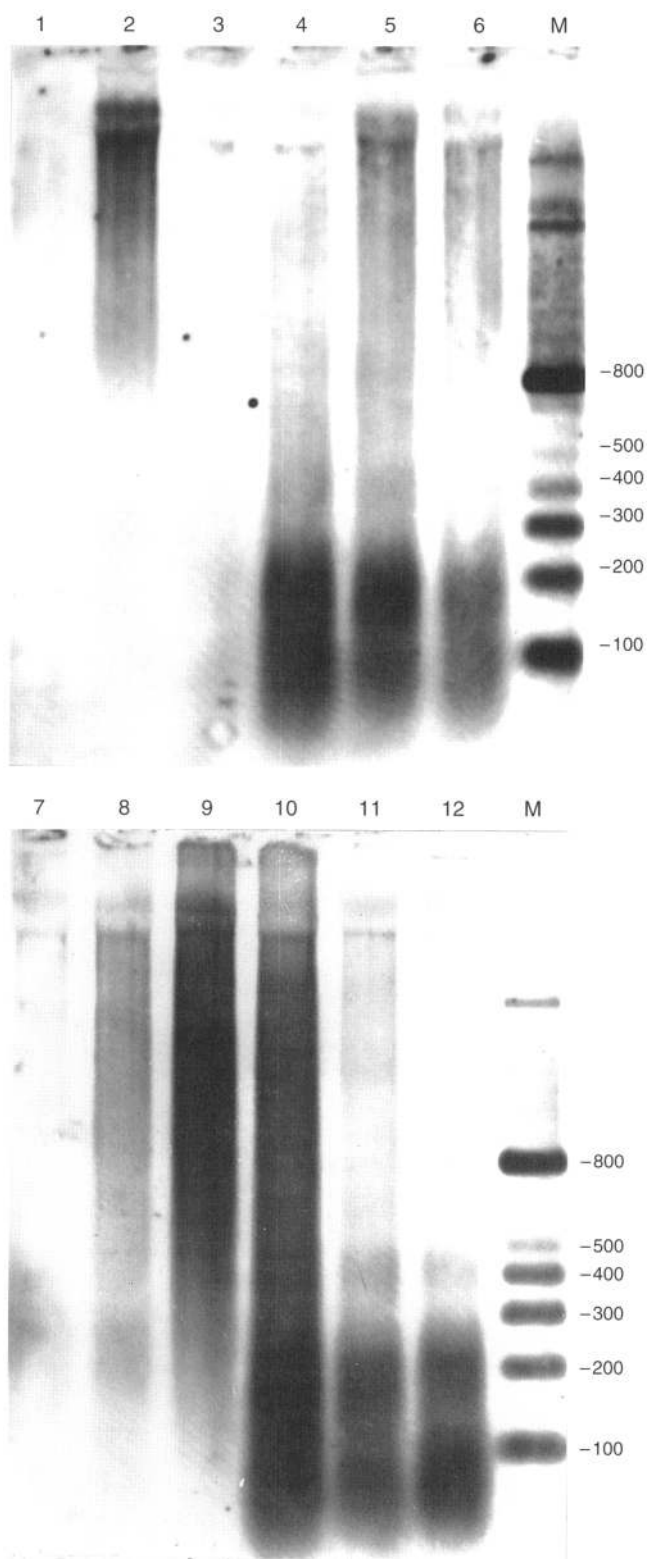


Fig. 1. The presence of oligonucleosomal-sized fragments in rat testicular DNA following treatment with GnRH antagonist (ANT: $125 \mu\text{g kg}^{-1} \text{day}^{-1}$ for 14 days) or methoxyacetic acid (MAA: 650 mg kg^{-1} , single dose). Lanes 1–3: ANT control; 4–6: ANT treated; 7–9: MAA control; 10–12: MAA treated. Samples were exposed to X-ray film for 6 min (lanes 1–6) or 2 min (lanes 7–12). M: markers (100 base pair ladder).

was confirmed by the quantitative comparison of dying germ cells at stage VII (Fig. 3b) detected by each method, which shows a close concordance between the two (Table 1). In this stage, however, germ cells with condensed chromatin that stained blue, that were not end-labelled, were also seen occasionally. Elongated, step-19 spermatid heads were still present at stages IX and X, located at the base of the epithelium and were biotin-positive (Fig. 3c). A summary of the cell degeneration data for this group is shown (Fig. 2b).

Twenty four hours after treatment with methoxyacetic acid extensive degeneration of spermatocytes had occurred. Pachytene spermatocytes at all stages were missing or exhibited abnormal morphology, being irregularly shaped and of various sizes. Nuclei were usually homogeneously stained, but some had condensed chromatin. A few preleptotene spermatocytes at stages VII and VIII were also PAS-positive. The situation was similar for the biotin-positive cells (Fig. 3d) and the same cells were identified as degenerating and at a similar frequency. Labelling of the majority of degenerating spermatocytes at stage XII–XIII can be seen (Fig. 3e). A section from a control testis at stage IX–X is also shown (Fig. 3f). The distribution of degenerating cells induced by methoxyacetic acid as identified by both methods is shown (Fig. 2c).

No testicular somatic cells were found to be either PAS- or biotin-positive in any of the groups.

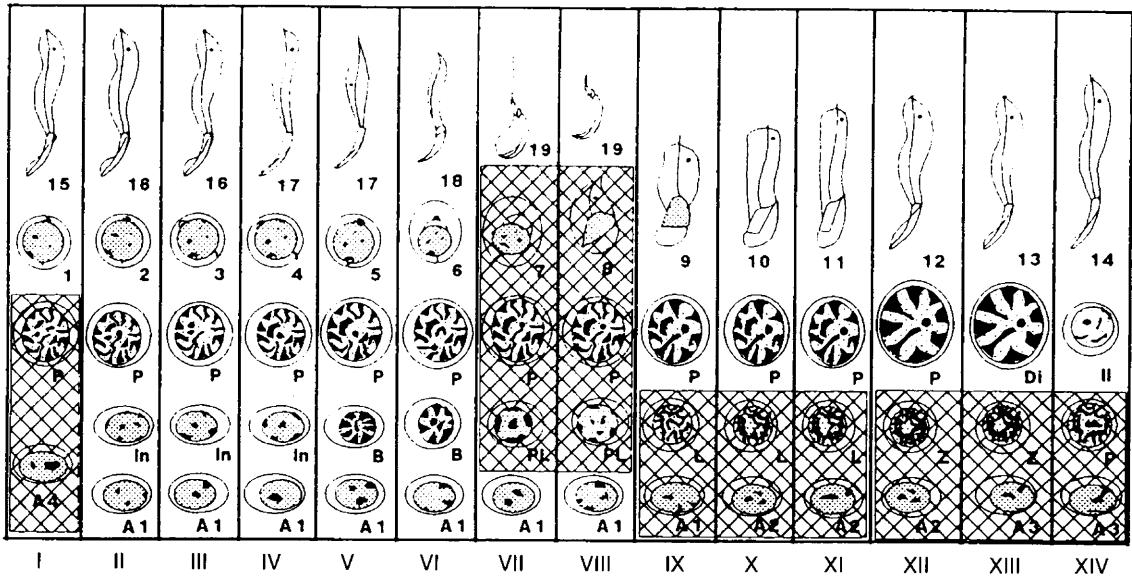
Discussion

The demonstration of electrophoretic ladders in testicular DNA from rats treated with a GnRH antagonist or methoxyacetic acid indicates that apoptosis occurred with both treatments. The fact that the bands of DNA were much clearer in the group treated with methoxyacetic acid is presumably due to the more extensive apoptosis caused by this treatment. Data from the group treated with the GnRH antagonist confirm the finding of Tapanainen *et al.* (1993) that apoptosis occurs in the testes. Whereas that report proposed that both interstitial cells and germ cells can be affected, the present work found no evidence of apoptosis in somatic cells. It is possible that this difference may result from the use of immature animals in the previous study.

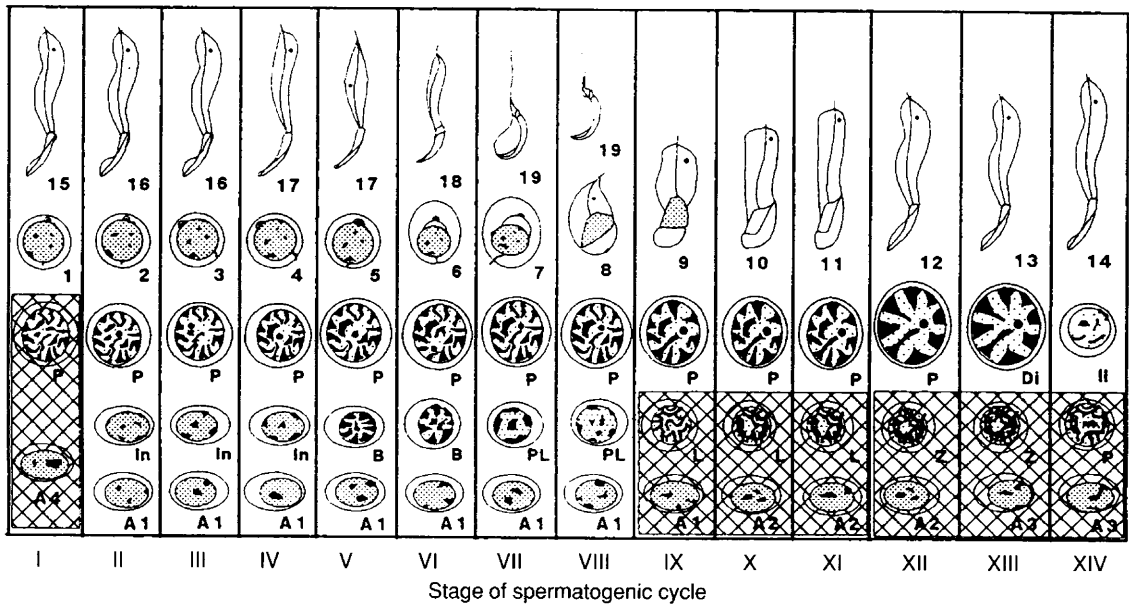
Billig *et al.* (1995) also demonstrated DNA ladders in the testes of immature rats and identified spermatocytes as the cell type principally affected. However, they were unable to show any effect of the treatment on adult animals but claimed a high rate of spontaneous apoptosis among meiotic spermatocytes as detected by *in situ* end-labelling (this identification has been questioned (Bartke, 1995)). The results obtained by Billig *et al.* (1995) with adult animals are in direct contrast to those presented here, which show a clear effect of treatment with a GnRH antagonist in inducing testicular apoptosis but only a very low rate of spontaneous, apoptotic cell death in the controls. One explanation for the discrepancy may lie in the shorter duration of treatment (4 days) used by Billig *et al.* Alternatively, the discrepancy may result from the much higher concentration of TdT used in that study ($1 \text{ U } \mu\text{l}^{-1}$), which, using the protocol presented here, was found to produce nonspecific labelling of nuclei at the periphery of the tubule.

(a)

Control: PAS-staining



Control: In-situ end-labelling



Stage of spermatogenic cycle

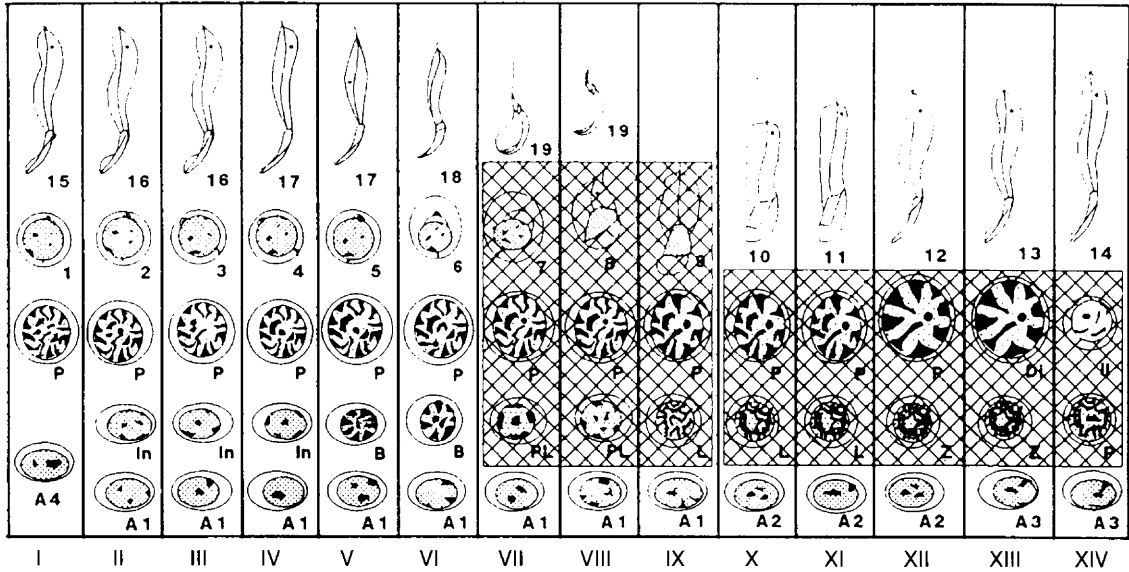
Fig. 2. Summary diagrams of the distribution of type of rat male germ cell undergoing degeneration (a) control and after treatment with (b) GnRH antagonist or (c) methoxyacetic acid as detected by staining with periodic acid–Schiff’s reagent and by end-labelling. Hatched areas represent the presence but not the number of degenerating cells.

The presence of a band of about 90 base pairs, in addition to the expected multiples of 185, observed from the DNA ladders is of interest. The reason for the presence of this band is not known, although it might result from the apoptotic degradation of cells with nucleosomal particles of different sizes. During spermatogenesis, the proteins that form the core of the nucleosomal particle, histones, are replaced by various transition proteins and finally by protamines. It is possible that a protein of this type could form a nucleosome particle that yielded smaller lengths of DNA during apoptosis.

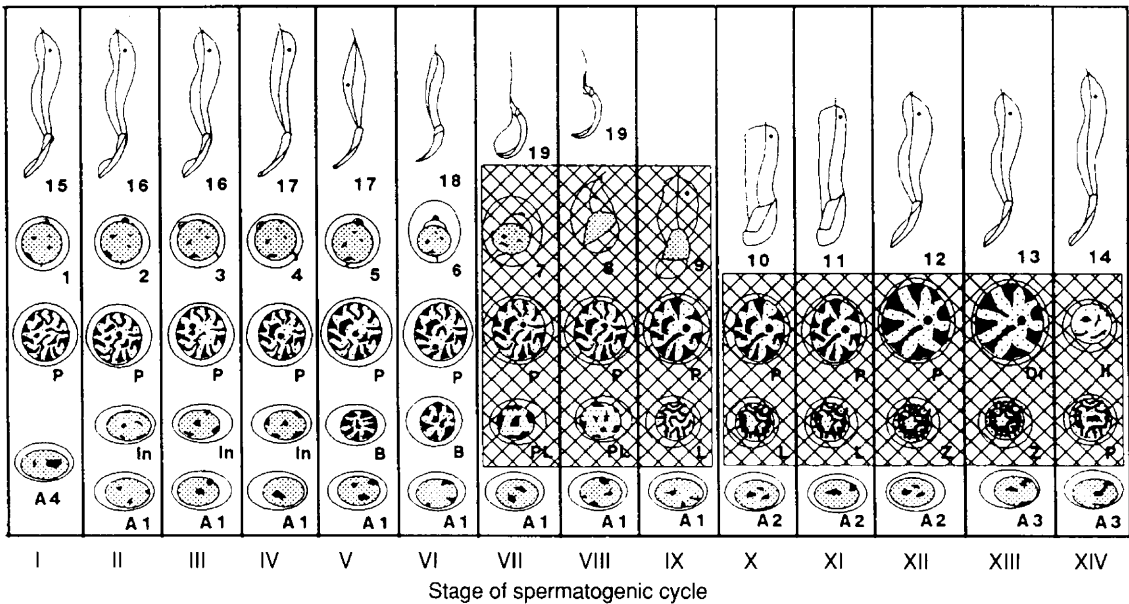
It is notable that the profile of dying cells in the control groups was so similar as determined by either PAS-staining or DNA end-labelling. Furthermore, the profile is in good agreement with the spontaneous germ cell death as described by Kerr (1992) in a study of 2063 tubular cross-sections from the testes of untreated rats. It has been well documented previously that in rats, testicular germ-cell loss can occur by spermatogonial deletion (Kerr, 1992) and this has been presumed to be apoptotic (Allan *et al.*, 1992). What is unusual in the present study is the variety of aberrant morphologies that

(b)

GnRH antagonist: PAS-staining



GnRH antagonist: In-situ end-labelling



Stage of spermatogenic cycle

Fig. 2. (Continued)

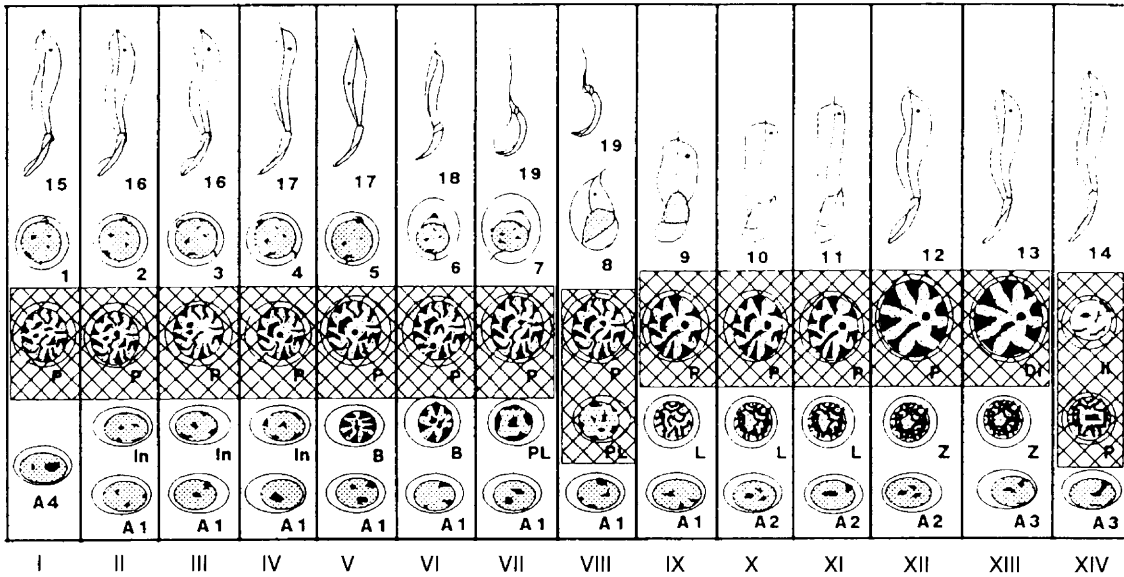
stained positive for biotin after end-labelling of the DNA. Conventionally, the morphology of apoptosis is defined by cellular shrinkage, chromatin and cytoplasmic condensation and fragmentation of the chromatin (Kerr *et al.*, 1972; Wyllie, 1987). This type of event was indeed observed in late-stage tubules of animals in the group treated with methoxyacetic acid. However, the most common characteristic of both spontaneous and GnRH antagonist induced germ cell death in the testis, as detected by PAS staining, tends to be cellular swelling and the development of decondensed, homogeneous chromatin. The presence of apoptotic ladders alongside this pattern (in the

GnRH antagonist group) coupled with the incorporation of biotinylated nucleotides into the nuclei of such cells suggest very strongly that testicular apoptosis can have a quite different morphology from that observed in somatic cells. The fact that this was not the case for most of the degenerating cells following treatment with methoxyacetic acid (at stages XII–XIV) also indicates that testicular apoptosis may take different forms depending on the mode of induction.

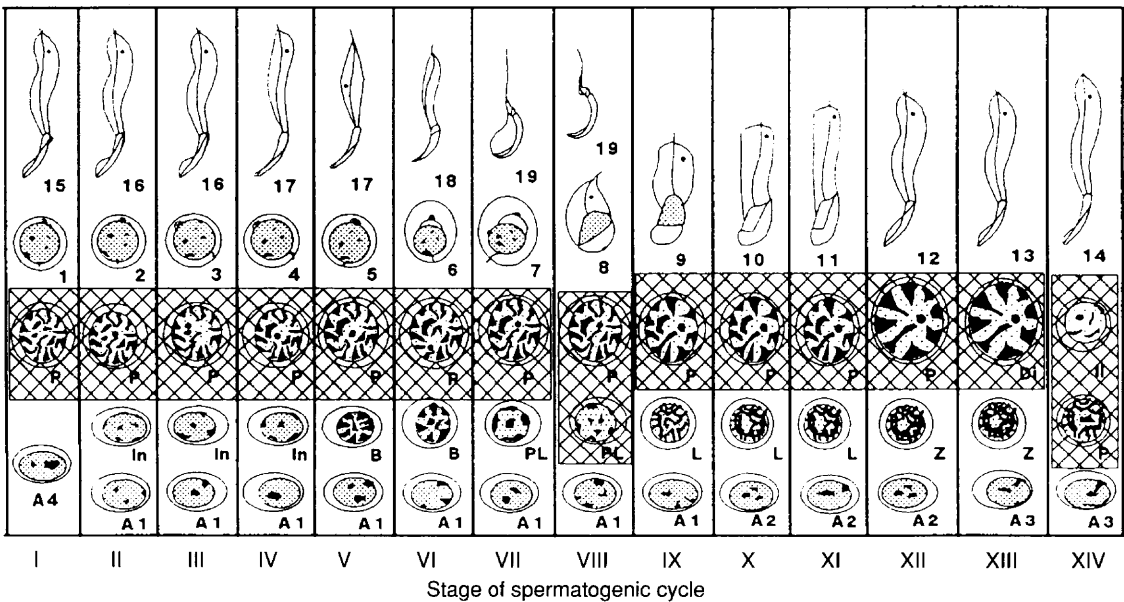
Biotin-stained sperm heads were observed at the base of the epithelium at stages VIII and IX of the group treated with the GnRH antagonist. These could also be seen in the

(c)

Methoxyacetic acid: PAS-staining



Methoxyacetic acid: In-situ end-labelling



Stage of spermatogenic cycle

Fig. 2. (Continued)

complementary PAS-stained sections, although not very clearly. It has previously been shown that treatment with a GnRH antagonist causes delayed spermiation, such that, some of the maturation-phase spermatids are not released into the lumen at the appropriate time (the end of stage VIII) (Sinha-Hikim and Swerdloff, 1993). As a result, these spermatids are (presumably) transported to the base of the epithelium and phagocytosed by the Sertoli cells. The biotinylation observed at that point could represent apoptosis, or, it could reflect degradation of the sperm DNA in Sertoli cell lysosomes, particularly since the time course for the appearance and

disappearance of these spermatid heads parallels that of the residual bodies.

The germ cell losses arising from treatment with the GnRH antagonist or methoxyacetic acid presumably result from different mechanisms, as suggested by the distinct ranges of cells affected and by the dissimilar effect of the treatments on gonadotrophins. The GnRH antagonist caused a drastic reduction in serum FSH and testosterone concentrations (to values below the sensitivities of the assays), whereas methoxyacetic acid had no significant effect. It is probable, therefore, that more than one type of stimulus can provoke an apoptotic

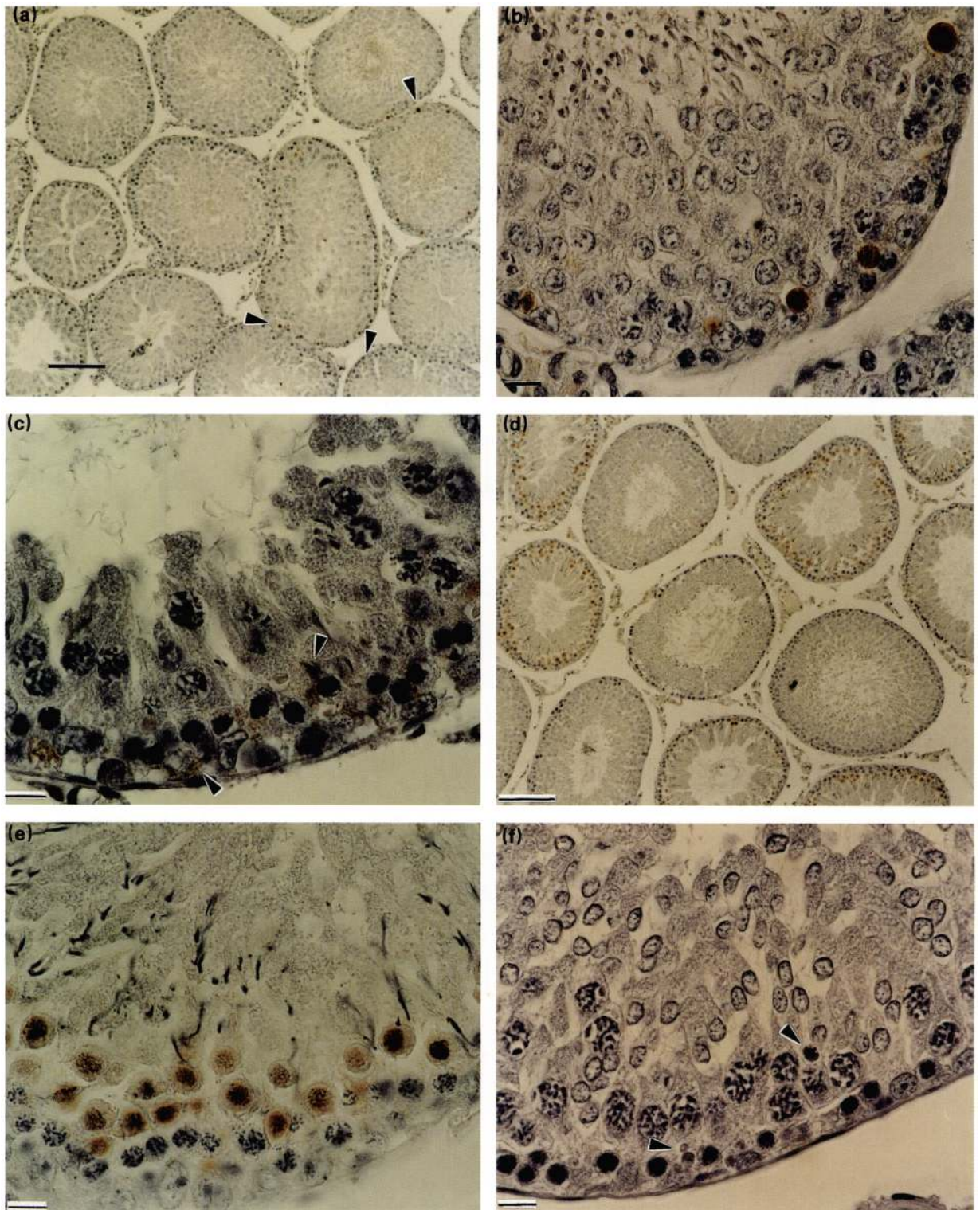


Fig. 3. End-labelled cells from rat testes. Tissue sections were reacted with biotinylated dUTP and terminal-deoxytransferase (TdT), and then hybridized with peroxidase-conjugated Extravidin and developed with diaminobenzidine. Labelled cells exhibit a brownish colour. (a) Low-power photomicrograph illustrating labelled cells from the testis of a rat treated with GnRH antagonist (examples arrowed). (b) Stage VII tubules showing GnRH antagonist-induced degenerating cells, presumed to be pachytene spermatocytes from their size and position. Note the relative homogeneity of the chromatin. (c) Tubule from a GnRH antagonist-treated rat with degenerating spermatid heads at the base of the epithelium (examples arrowed). (d) Low-power photomicrograph illustrating labelled cells from the testis of a rat treated with methoxyacetic acid. Considerably more cells are labelled than in the GnRH antagonist-treated testis. (e) Labelling of the majority of the degenerating spermatocytes in a stage XII/XIII tubule of a rat treated with methoxyacetic acid. Note the clumpy appearance of the chromatin compared with that in (b). (f) Section from a control testis at stage IX-X. Residual bodies can be seen in the epithelium and are not end-labelled (examples arrowed). Scale bars represent 100 μm ((a) and (d)) and 10 μm ((b), (c), (e) and (f)).

response in the testis and, hence, that there is more than one pathway in the testis leading to this type of physiological cell death. Since this cell death would involve certain specific gene activity, molecular investigations may help determine whether the different pathways possess common mechanisms.

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