

Correlation between DNA synthesis in the second, third and fourth generations of spermatogonia and the occurrence of apoptosis in both spermatogonia and spermatocytes

J. Blanco-Rodríguez¹, C. Martínez-García¹ and A. Porras²

¹Department of Cell Biology, School of Medicine, Valladolid University, Ramón y Cajal, 7, 47005 Valladolid, Spain; and ²Department of Biochemistry and Molecular Biology, School of Pharmacy, (CSIC/UCM), Madrid, Spain

In the seminiferous epithelium, both DNA synthesis and apoptosis occur at equivalent stages in various species, with apoptosis taking place mainly at the same stages as DNA replication in the second, third and fourth spermatogonial generations. As preservation of the cellular associations found at these stages may have some functional significance, it is important to determine whether there is a correlation between these cellular events. In this study, pairs of immunoperoxidase-stained adjacent testis sections from rats, mice, rabbits and cats in which either bromodeoxyuridine incorporated into the newly synthesized DNA strand (BrdU labelling) or DNA 3' end labelling

of the apoptotic DNA fragments (TUNEL assay) were detected were compared. In addition, both events were analysed in double-labelled sections. These two methods revealed a clear correlation between the occurrence of DNA replication in the second to fourth generations of spermatogonia and most physiological apoptosis taking place in both spermatogonia and spermatocytes in the three different mammalian orders (Rodentia, Lagomorpha and Carnivora). This correlation may result from the synchronization of mitotic spermatogonial and meiotic spermatocyte cell cycle checkpoints operating at these stages.

Introduction

Spermatogenesis is a precisely controlled and timed process comprising the mitotic proliferation of spermatogonia, the meiotic divisions of spermatocytes and the differentiation of haploid spermatids to spermatozoa. Consequently, cross-sections of seminiferous tubules are characterized by the appearance of typical cellular associations of several germ cell progenies, each at a different stage of differentiation, known as stages of the spermatogenic cycle. Although the duration of the spermatogenic cycle, as well as the number and pattern of cell associations, vary greatly among species, such associations have been described for most mammals (Leblond and Clermont, 1952; Oakberg, 1956; Swierstra and Foote, 1963; Ibach *et al.*, 1976; Böhme and Pier, 1986; Johnson *et al.*, 1990; Komatsu *et al.*, 1996). Nevertheless their biological significance is unknown.

The seminiferous epithelium is one of the most productive self-renewing systems in the body in which apoptosis is also an important phenomenon (Print and Loveland, 2000). Classical data on germ cell degeneration during spermatogenesis in small rodents show that cell death is common among the second to fourth gener-

ations of committed spermatogonia around their mitotic peaks (for review, see Blanco-Rodríguez, 1998). Induction of germ cell apoptosis in rats by various stress stimuli revealed that most induced cell deaths of both spermatogonia and spermatocytes take place at these stages (Blanco-Rodríguez and Martínez-García, 1997, 1998). More recently, stage-specific analyses of DNA synthesis and germ cell apoptosis in rabbits (Blanco-Rodríguez, 2002a) and cats (Blanco-Rodríguez, 2002b) have shown that DNA synthesis, as well as most physiological cell deaths in these animals also occur at stages equivalent to those in small rodents. As the preservation of these cellular associations in three different orders of mammal (Rodentia: mice and rats; Lagomorpha: rabbits; and Carnivora: cats) may have a functional significance, the present study investigated whether there is a correlation between DNA synthesis and apoptosis at these stages in these four species. Pairs of immunoperoxidase-stained adjacent sections were compared to detect either DNA synthesis or apoptosis, which allowed a precise diagnosis of the type of cell and staging for accurate quantification. Double-fluorescent-labelled sections were also analysed, which proved very useful for illustrating and quantifying coincidences of both events at the same discrete tubule areas. Tissues from rats, mice, rabbits and cats were processed at the

Email: jblanco@med.uva.es

same time to avoid differences arising from technical procedures.

Materials and Methods

Animals and tissue preparation

The testes from four different species, Wistar rats (aged 3 months), Swiss mice (aged 3 months), New Zealand rabbits (aged 9 months) and cats (*Felis domesticus*; aged 1 year), were analysed. Three animals of each species, housed under conventional, controlled standard conditions, were used. Testicular tissues from the rabbits and cats were derived from paraffin wax-embedded tissues previously used for the above mentioned stage-specific analyses of DNA synthesis and germ cell apoptosis in these animals (Blanco-Rodríguez, 2002a,b). Every animal was injected with 100 mg bromodeoxyuridine kg^{-1} body weight (BrdU; Sigma Chemical Co., St Louis, MO) 1 h before it was killed. After anaesthesia by sodium pentobarbital (70 mg kg^{-1} body weight, i.p.), testes were prepared by making an incision from the proximal to the distal pole. Pieces of testis parenchyma were immersion-fixed in 4% (v/v) formaldehyde in 0.1 mol phosphate-buffer (pH 7.4) l^{-1} , embedded in paraffin wax and cut into cross-sections. Pairs of adjacent sections (5 μm thick) were mounted on TESPA (3-aminopropyl-tri-ethoxysilane)-coated slides (Sigma). Paraffin wax-embedded sections were dewaxed, hydrated and processed for the subsequent detection of either DNA synthesis or apoptosis.

BrdU DNA labelling

The first section of each pair was used for BrdU detection. BrdU retrieval was performed by incubating the sections for 20 min in 0.1% (w/v) trypsin (Sigma) in 0.1% (w/v) CaCl_2 (pH 7.8) at 37°C, and by DNA denaturation in 2 M HCl for 1 h at 37°C before the antibody reaction. BrdU was detected using an anti-BrdU antibody (Dakopatts, Glostrup; 10.5 $\mu\text{g ml}^{-1}$) and an avidin-biotin complex (ABC)-based method. The horseradish peroxidase ABC reaction was performed using the Santa Cruz immunoperoxidase staining kit (ABC reagents; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), according to the manufacturer's protocol. The endogenous peroxidase was quenched by 15 min incubation in 2% (v/v) hydrogen peroxide in PBS.

DNA 3' end labelling of apoptotic cells

The second section of each pair was used for TUNEL staining of apoptotic DNA fragmentation, using the Oncor ApopTag non-radioactive detection kit (Oncor Inc., Gaithersburg, MD). Hydrated sections were treated with 20 μg proteinase K ml^{-1} (Boehringer-Mannheim, Mannheim) for 15 min at room temperature and then

incubated with a reaction mixture for DNA 3' end labelling for 1 h at 37°C in a humidified chamber. The reaction mixture contained terminal transferase reaction buffer, digoxigenin-deoxyuridine triphosphate (DIG-dUTP) and terminal deoxynucleotidyl transferase (TdT), and the reaction was performed following the supplier's guidelines. DNA strand breaks were revealed after incubation with anti-DIG antibody conjugated to peroxidase at room temperature for 30 min, and the subsequent detection of enzyme activity using 3,3'-diaminobenzidine.

Double fluorescent labelling of BrdU and DNA apoptotic fragments

For double labelling of DNA synthesis and apoptosis, a protocol similar to that for immunoperoxidase detection was used. Fluorescent labelling of DNA fragmentation was performed using the fluorescein Promega apoptosis detection system (Promega, Madison, WI) following the supplier's guidelines with minor modifications to allow subsequent BrdU labelling of the same testis section. In brief, after incubation with the TdT and the nucleotide mix, the slides were rinsed in $2 \times \text{SSC}$ for 15 min and then in distilled water. DNA denaturation was performed before incubation with the anti-BrdU antibody as described above. The anti-BrdU antibody was detected with donkey anti-mouse Alexa Fluor 546 (Molecular Probes Europe BV, Leiden) diluted 1:1000, incubated for 1 h at room temperature. The sections were mounted with Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA).

Image analysis and quantification

Sections used for analysis of apoptosis and BrdU labelling were counterstained in periodic acid-Schiff (PAS)-cresyl violet for accurate identification of type of cell and stage under a bright-field microscope. In addition, special attention was paid to the observation of mitotic figures of spermatogonia in these counterstained sections. Determination of stage was carried out at $\times 630$ magnification, using a $\times 63$ (1.4 numerical aperture) planapochromatic oil immersion objective (Carl Zeiss), following the criteria proposed by Leblond and Clermont (1952) for rats, Oakberg (1956) for mice, Swierstra and Foote (1963) for rabbits, and Böhme and Pier (1986), with minor modifications (Blanco-Rodríguez, 2002b), for cats. TdT-mediated dUTP nick-end labelling (TUNEL)-stained cells observed in circular or near-circular tubule sections were identified according to their morphology and position, following previously established criteria (Blanco-Rodríguez and Martínez-García, 1996). All the labelled cells were recorded except for dying elongated spermatids. For each animal, ten non-consecutive sections were chosen at random. For quantification of stage-specific apoptosis a total of

30 immunoperoxidase-stained tubules were studied at each stage and counts were expressed as a ratio to the number of Sertoli cell nucleoli.

On the basis of the number and distribution of fluorescent cells, as well as on the tubule profiles, and other morphological details revealed by either DAPI fluorescence or phase contrast optics, seminiferous tubules of double-labelled sections were classified as tubules (i) showing or (ii) not showing BrdU staining. Seminiferous tubules showing BrdU staining were subclassified into those at which the second to fourth spermatogonial generations replicate DNA or tubules with other replicating spermatogonia. Apoptotic cells appearing in these tubules were quantified in ten non-consecutive sections from each animal chosen at random. The filters used to visualize fluorescein (excitation: 450–490 nm; emission: 515–565 nm), Alexa 546 (excitation: 546 nm; emission: 590 nm) and DAPI (excitation: 365 nm; emission: 397 nm) were from Zeiss. The illumination source was a 50 W mercury arc lamp.

Images of representative fields were acquired using a computer-assisted (Spot, RT Color; Diagnostic Instruments Inc., Sterling Heights, MI) cooled charge-coupled device (1520 by 1080 pixels) on a Zeiss Axiophot light microscope (Zeiss Inc.). Digital images were processed using Adobe PhotoShop 6.0. (Adobe Systems Inc.).

Results

Analysis of BrdU-labelled and TUNEL-stained contiguous sections

PAS–cresyl violet counterstaining allowed accurate staging and identification of type of cell in most cases, according to the nucleus morphology, size and position in the epithelium. Quantification of immunoperoxidase TUNEL-stained cells in every second member of the pairs of sections, processed for the four species at the same time, confirmed or closely reproduced previous findings. Nevertheless, to make the comparison among the various species analysed easier, these data are shown together (Fig. 1). Apoptosis is stage-specific, and spermatogonial and spermatocyte death usually occur at those stages at which differentiating spermatogonia of the second, third and fourth generations divide (Fig. 1a). Dying cells were mainly: (i) early zygotene and ending pachytene spermatocytes, at the same stage as spermatogonia of the second generation (stage XII for rats; stage X for mice; stage 2 for rabbits; and stage VIIa for cats; Fig. 1b); (ii) spermatocytes at the zygotene–pachytene transition, and those undergoing the meiotic divisions died at the same stage as the third generation of spermatogonia (stage XIV for rats; stage XII for mice; stage 4 for rabbits; and stage VIII for cats; Fig. 1c); (iii) starting pachytene spermatocytes coinciding with the fourth generation of spermatogonia (stage I for rats and mice; stage 5 for rabbits; and stage Ia for cats; Fig. 1d). In contrast, cell

death was rarely observed at other developmental steps (Fig. 1a).

Importantly, in addition to confirming previously established data, the comparison between BrdU-labelled and TUNEL-stained contiguous sections revealed that, in every animal, most spermatogonia and spermatocytes died not only at the same stage, but also at the same tubule regions as those in which the second, third and fourth generations of spermatogonia underwent DNA replication (Fig. 2). Scattered mitotic figures also appeared at these regions, indicating that completion of DNA synthesis was immediately followed by spermatogonial divisions (Fig. 2b).

Double immunofluorescent detection of BrdU-labelled and TUNEL-stained germ cells

Although the above-described strategy enabled precise identification of types of germ cell and spermatogenic stage in every species, the observation of the same tubule in two different sections, localizing DNA replication in one and subsequently quantifying apoptosis in the contiguous section, proved extremely difficult. Therefore, although the comparison of pairs of either BrdU or TUNEL immunoperoxidase-stained sections allowed observation of the correlation between the occurrence of DNA synthesis in certain spermatogonial generations and apoptosis, double fluorescent labelling of both processes in the same testis section was performed to demonstrate this correlation more clearly.

The nuclei of the first generation of DNA replicating spermatogonia were difficult to distinguish in these fluorescent-stained sections, as a result of their very low number and position among spermatocytes undergoing premeiotic DNA replication. Nevertheless, these tubules were easily recognized as they showed the highest numbers of BrdU-labelled cells, these cells being in close proximity to one another (Fig. 3). The presence of round spermatids and the heads of the spermatozoa lining the tubule lumen at the top of the epithelium were also characteristic features easily observed in these tubule sections. Likewise, on the basis of the number and distribution of DNA replicating and apoptotic cells, as well as on the grounds of the tubule profiles and other morphological details revealed by either DAPI fluorescence or contrast phase optics, it was possible to distinguish between tubule sections at which the second to fourth spermatogonial generations replicate DNA, and other tubule sections. Thus, tubules corresponding to the fifth and sixth generations of DNA replicating spermatogonia also showed a high number of BrdU-labelled cells plus round spermatids. However, labelled nuclei of spermatogonia were separated from one another, and the head of the elongated spermatids was embedded in the epithelium. In these tubules showing the highest numbers of BrdU-labelled cells, and corresponding to the first, fifth

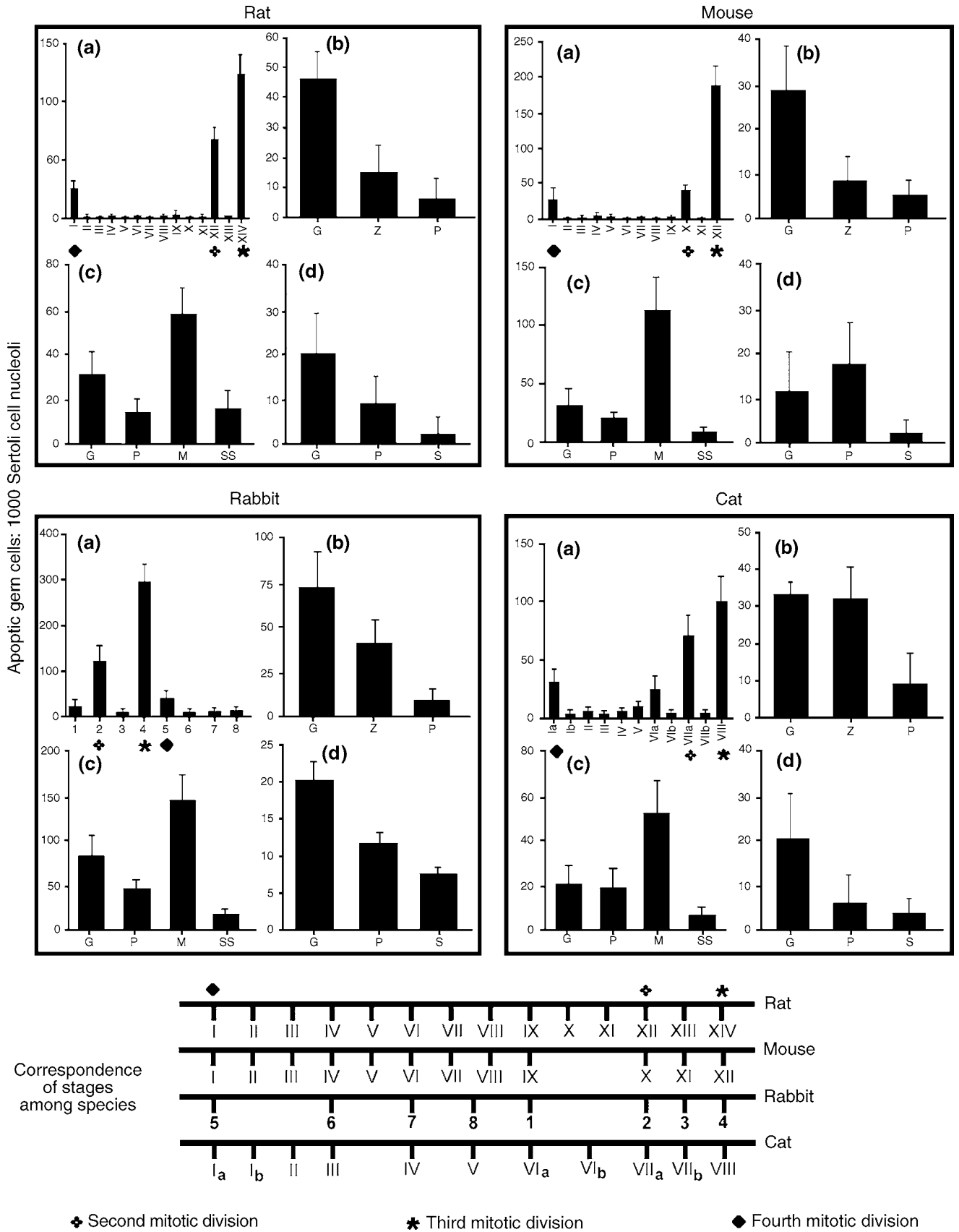


Fig. 1. For legend see facing page.

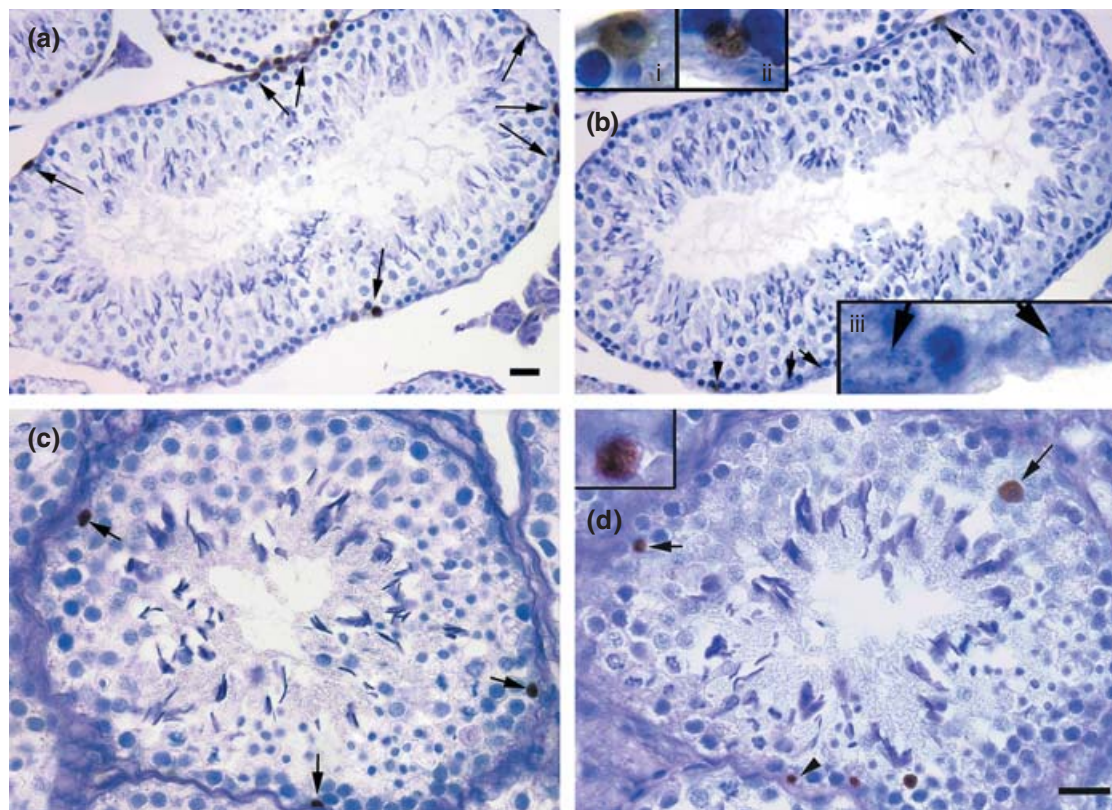


Fig. 2. Immunoperoxidase detection of DNA synthesis and apoptosis in pairs of adjacent sections. Periodic acid–Schiff (PAS)–cresyl violet counterstaining. (a,b) Stage X seminiferous tubule from a mouse (equivalent to stage XII for rats; stage 2 for rabbits; and stage VIIa for cats). (a) Bromodeoxyuridine (BrdU) labelling. Arrows indicate committed spermatogonia of the second generation. (b) TdT-mediated dUTP nick-end labelling (TUNEL) staining of the same tubule at the adjacent section. Long arrow indicates a spermatogonium (its globular shape and localization close to the basal membrane are visible in inset (i)). Arrowhead indicates a zygotene spermatocyte as judged by the preservation of chromosome pairing and localization (see inset (ii)). Short arrows indicate prophase spermatogonia (see inset (iii)). (c,d) Stage 4 (equivalent to XIV for rats; stage XII for mice; and stage Ia for cats) seminiferous tubule from a rabbit. (c) Arrows indicate committed spermatogonia of the third generation showing BrdU labelling. (d) TUNEL staining of the consecutive tubule section. Long arrow indicates a metaphase I spermatocyte, the short arrow indicates an early pachytene spermatocyte (see inset) and the arrowhead indicates a spermatogonium. Scale bars represent 50 μm .

and sixth spermatogonial generations, as well as in those with no BrdU labelled cells, TUNEL-stained cells were very rarely observed. In contrast, most apoptotic cells were found in tubules showing a low to medium number of BrdU-labelled cells (Fig. 3). The absence of round spermatids in most of these tubules indicated that replicating spermatogonia corresponded to the second

(when pachytene spermatocytes were present) or the third generation (when dividing spermatocytes were present). The remaining tubules in this group, showing BrdU labelling, apoptotic cells and round spermatids, corresponded to tubules with replicating spermatogonia of the fourth generation, as indicated not only by the lower number of replicating spermatogonia compared

Fig. 1. Quantitative data for TdT-mediated dUTP nick-end labelling (TUNEL)-stained germ cells confirming previous findings are given together to allow comparison among the different species analysed. Numbers of apoptotic cells are expressed as a ratio to Sertoli cell nucleoli. Values are mean \pm SEM. (a) Total number of labelled cells at every stage. Most cell deaths were observed coinciding with mitotic peaks of spermatogonia of the second to fourth generations (stages XII, XIV-I for the rat, Leblond and Clermont, 1952; X, XII-I for the mouse, Oakberg, 1956; 2, 4–5 in the rabbit, Swierstra and Foote, 1963; and VIIa, VIII-Ia in the cat, Böhme and Pier, 1986, with minor modifications Blanco-Rodríguez, 2002b). Germ cell associations at these stages were equivalent for every species. (b–d) Quantification of the different types of apoptotic germ cell at these stages. (b) G: spermatogonia of the second generation; Z: early zygotene spermatocytes; P: ending pachytene spermatocytes. (c) G: spermatogonia of the third generation; P: spermatocytes at the zygotene–pachytene transition; M: metaphase I spermatocytes; SS: secondary spermatocytes. (d) G: spermatogonia of the fourth generation; P: very early pachytene spermatocytes; S: round spermatids.

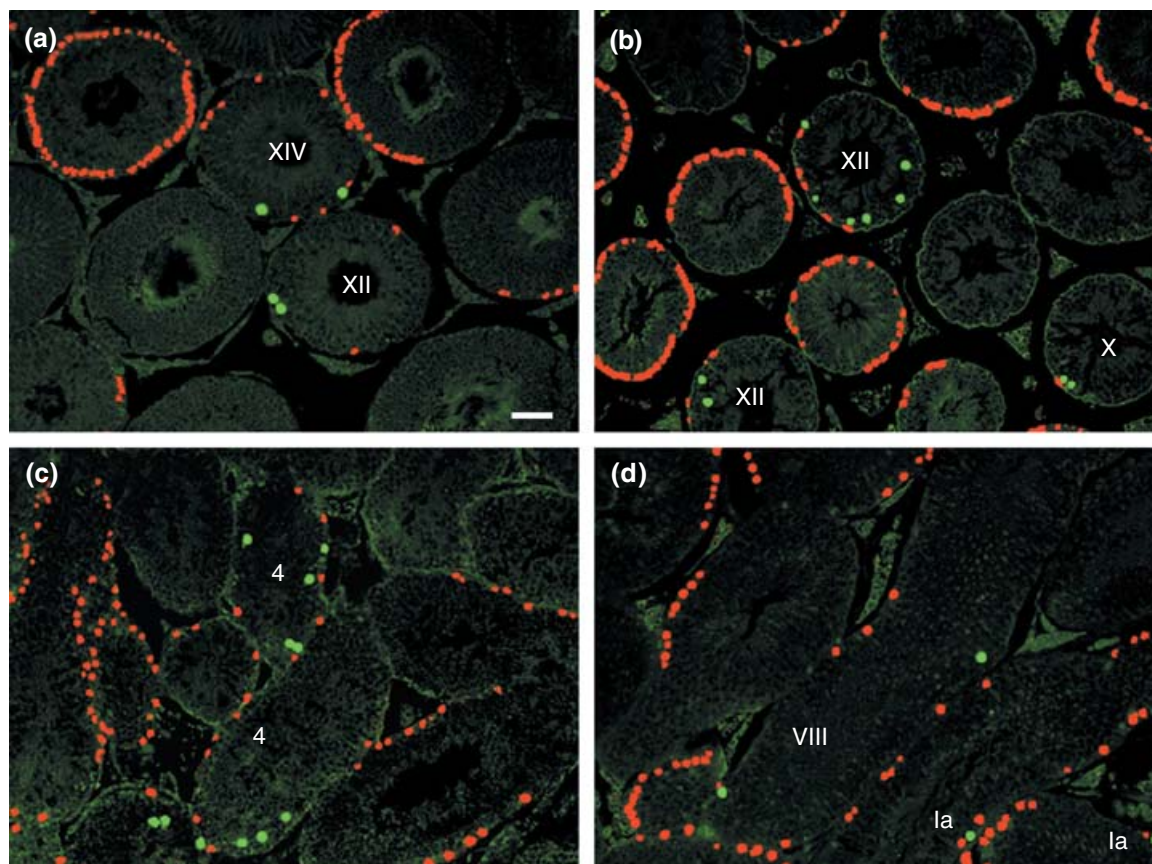


Fig. 3. Double immunofluorescent detection of bromodeoxyuridine (BrdU) labelling (red) and TdT-mediated dUTP nick-end labelling (TUNEL) staining (green) in testis sections from (a) a rat, (b) a mouse, (c) a rabbit and (d) a cat. Tubules with the highest number of BrdU-labelled cells correspond to those showing replicating spermatocytes and spermatogonia of the first generation. In every species shown here, apoptotic cells appear together with tubules with a low to medium number of BrdU-labelled cells. (a) The rat section shows two of these tubules in which apoptotic cells appear with replicating spermatogonia of the second (XII) and the third generation (XIV). (b) In the mouse section, apoptotic cells are observed with labelled spermatogonia of the second (X) and third (XII) generations. (c) In the rabbit section, apoptotic cells are in tubules showing DNA synthesis in spermatogonia of the third generation (4). (d) In the cat section, apoptotic cells are with spermatogonia of the third (VIII) and fourth (Ia) generations. Scale bar represents 100 μm .

Table 1. Total average number of apoptotic cells per testis section observed in each species and percentages of these cells found in the different types of tubule

Species	Total average number	BrdU-stained tubules		Tubules not stained with BrdU (%)
		Second to fourth spermatogonial generations (%)	Other BrdU-stained tubules (%)	
Rat	35.6 \pm 2.3	97.1 \pm 1.7	2.1 \pm 1.9	0.6 \pm 0.2
Mouse	30.7 \pm 1.8	95.8 \pm 2.1	3.1 \pm 2.1	0.9 \pm 0.1
Rabbit	56.3 \pm 3.6	93.3 \pm 3.1	3.9 \pm 0.8	1.7 \pm 0.3
Cat	52.4 \pm 4.1	91.8 \pm 3.6	5.3 \pm 1.7	1.9 \pm 0.9

Values are mean \pm SEM.
BrdU: bromodeoxyuridine.

with those corresponding to the fifth and sixth spermatogonial generations, but also by the distribution pattern of the heads of the elongated spermatids and other morphological features shown by DAPI or contrast phase optics. Quantification of apoptosis in these sections

confirmed the findings in the study of pairs of PAS–cresyl violet counterstained sections, evidencing that a large percentage of apoptotic cells were located at tubules showing spermatogonia of the second, third or fourth generations undergoing DNA replication (Table 1).

Discussion

The seminiferous epithelium is a highly proliferative tissue, with mitotic peaks of committed spermatogonia occurring at specific stages along the length of the tubules. It is assumed that the number of germ cells should fit the capability of the Sertoli cells to support them and that this number is adjusted by cell death when proliferation of differentiating spermatogonia begins (for a review on the significance of germ cell apoptosis, see Blanco-Rodríguez, 1998). Consistent with this idea, cell death usually occurs at the same stages at which the second, third and fourth generations of committed spermatogonia divide. The cellular associations found at these stages are preserved in different orders of mammal (Oakberg, 1956; Russell and Clermont, 1977; Huckins and Oakberg, 1978; Blanco-Rodríguez, 2002a,b), indicating that they may have a functional significance. The two methods used in the present study provide evidence of a clear relationship between DNA replication in these generations of spermatogonia and most physiological apoptosis occurring in both spermatogonia and spermatocytes. Therefore, as checkpoints monitor DNA integrity before S phase, during replication and before mitosis (O'Connell *et al.*, 2000; Bartek and Lukas, 2001), and apoptosis is often related to the activity of checkpoints controlling cell cycle progression (Hickman *et al.*, 2002), the evidence that spermatogonial apoptosis mainly occurs within the regions in which the second, third and fourth generations of spermatogonia replicate DNA indicates that these cell deaths may be related to spermatogonial cell cycle checkpoints.

The high sensitivity of spermatocytes during zygotene and metaphase has been well established for rats (Kerr, 1992; Blanco-Rodríguez and Martínez-García, 1998), mice (Oakberg, 1956; Roosen-Runge, 1973) and humans (Johnson *et al.*, 1983, 1984), and, more recently, for rabbits (Blanco-Rodríguez, 2002a) and cats (Blanco-Rodríguez, 2002b). Importantly, localization and identification of apoptotic cells and the subsequent observation of the same tubule in the contiguous section not only confirmed previous findings, but also provided evidence of a clear correlation between the death of these cells and DNA synthesis in the aforementioned spermatogonial generations: that is, (i) apoptosis in early zygotene and ending pachytene spermatocytes mainly occurred within the same tubule areas in which the second generation of differentiating spermatogonia underwent both apoptosis and DNA replication; (ii) apoptosis of spermatocytes at the zygotene–pachytene transition and at metaphase I is found at the same areas in which spermatogonia of the third generation undergo apoptosis and DNA replication; and (iii) very early pachytene spermatocytes are found dying with both replicating and apoptotic spermatogonia of the fourth generation.

Detection of the phosphorylated form of histone H2AX (γ -H2AX) at sites of DNA double strand breaks

(DSBs) in spermatocyte squashes indicates that DSBs responsible for meiotic recombination take place during the leptotene–zygotene stage, and that DSBs also appear covering the unpaired X chromosome at the zygotene–pachytene transition before sex body formation (Mahadevaiah *et al.*, 2001). Checkpoint protein kinases, such as ATR and ATM, phosphorylate γ -H2AX in response to DSBs (Burma *et al.*, 2001; Ward and Chen, 2001) and γ -H2AX may play a critical role in the recruitment of repair factors (Paull *et al.*, 2000). Considering these data together, it is possible that visualization of apoptotic spermatocytes at the developmental steps which follow the appearance of γ -H2AX after programmed DSBs might be the consequence of DNA damage checkpoints operating at these stages. As mitotic divisions of spermatogonia are synchronized to meiotic divisions of spermatocytes, the coincidence of apoptotic spermatogonia and spermatocytes at the same tubule sections might also indicate that this synchronization is carried out through the synchronized control of mitotic and meiotic checkpoints. This synchronization might depend upon signals delivered from the Sertoli cells in response to hormones or to signals secreted by the germ cells themselves at specific stages. This hypothesis is consistent with the fact that in every species DNA replication in premeiotic spermatocytes occurs at the same time as DNA replication in the first generation of committed spermatogonia, whereas the division of the third generation coincides with metaphase I spermatocytes, at which a spindle assembly checkpoint seems to operate (Page and Orr-Weaver, 1997; Eaker *et al.*, 2002). Cells can activate checkpoints, but also tolerance pathways that facilitate survival (Verkade *et al.*, 2001). This may be the case with other generations of spermatogonia. Thus, under physiological conditions, overriding checkpoints during the first, fifth and sixth mitotic divisions of spermatogonia may be reasonable, taking into account that there is either a low number of differentiating cells or that their numbers have already been adjusted to those that Sertoli cells can support. In addition, in every species the first, fifth and sixth spermatogonial generations are associated with mid-pachytene spermatocytes.

Studies aiming to provide a global explanation for the architectural organization of the seminiferous epithelium have never been performed. This comparative study among members of three different orders of mammal shows a clear correlation between the cellular events of DNA synthesis (subject to cell cycle checkpoint control) and apoptosis (often linked to such checkpoints), and offers a possible explanation for the typical germ cell associations characterizing the spermatogenic stages in mammals.

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