

Original Article

Seminiferous epithelium cycle staging based on the development of the acrosome in ram testis

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Abstract: Testicular histopathology is considered the most sensitive and reliable method to detect the effects of chemicals on sperm production. To carry out a sensitive examination of testicular histopathology and interpret the changes require knowledge of spermatogenic stages. Spermatogenic staging based on acrosome development during spermiogenesis is conventionally performed in animal species routinely used for research and toxicity testing. In contrast, small ruminants, such as sheep and goats, are rarely used as animal models to evaluate toxicity in male reproductive organs. To the best of our knowledge, a comparable spermatogenic staging system in rams has not yet been fully characterised. Hence, this study aimed to adapt the existing spermatogenic staging based on acrosome development in bull testes to fit the seminiferous epithelium cycle of ram testes. The results show that spermatogenic staging based on acrosome development in bull testes can, with slight modifications, be efficiently used for the staging of ram testes. (DOI: 10.1293/tox.2021-0025; *J Toxicol Pathol* 2021; 34: 331–338)

Key words: ram, spermatogenesis, staging, acrosome development, spermiogenesis

Introduction

Spermatogenesis is a highly coordinated cyclic process that encompasses different cell associations called stages¹. Two approaches exist in mammals to characterise the seminiferous epithelium cycle stages. One classification system uses spermiogenesis as a reference point and usually consists of eight stages²; the other uses the developmental status of the acrosomic system during spermiogenesis³. The latter has been proven to be more precise and objective³ and, hence, is routinely utilised to investigate the effects of hormones and substances on spermatogenesis^{3–5}. The testicles of rat⁶, mouse⁷, and dog^{8,9} are commonly investigated based on the latter system, as these are the most frequently used species in research as well as in toxicology testing⁸. Nevertheless, seminiferous staging on the basis of acrosome development during spermiogenesis has been described in many other species, including monkeys^{10–14}, guinea pigs¹⁵, hamsters^{16,17},

gerbils¹⁸, minks¹⁹, pigs^{20,21}, opossums²², armadillos^{23,24}, quails²⁵, turtles²⁶, and bulls³.

Sheep have been used as an animal model for reproductive issues and technologies, vaccine development, and research into the pathology of respiratory disease and as a surgical model, from bone and wound repair to heart pathology²⁷. In addition, sheep are the most common farm animal species used to study endocrine disruption²⁸. Several studies have described spermiogenesis and spermatogenesis in rams. The staging systems for rams have been defined using spermiogenesis as a reference point^{2,29}. The morphology, proliferation, and differentiation of undifferentiated spermatogonia in ram testes have also been described³⁰, and the development of the ram acrosome has been reported³¹. Nonetheless, to the best of our knowledge, the seminiferous epithelium cycle has not been investigated in the context of spermatid development.

This study aimed to adapt the spermatogenic staging based on acrosome development in bull testes³ to ram testes and to describe spermatogenesis and the cellular composition of the nine distinguishable stages of the spermatogenic cycle in ram testes. In addition, we provide clear, illustrative photomicrographs of the individual stages in sections of Bouin's-fixed, periodic acid-Schiff-stained, and haematoxylin-counterstained (PAS-H) ram testes.

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Materials and Methods

Animals

Six 11-month-old Istrian Pramenka rams, an indigenous Slovenian sheep breed, were used in this study. The animals were born and raised in a sheepfold at the Vremščica Infrastructure Centre for Sustainable Recultivation (ICSR) of the University of Ljubljana, Veterinary Faculty, in Slovenia. The rams were kept in a stable, under natural photoperiod, with temperature conditions varying from 6°C to 15°C and relative air humidity between 45% and 55%. They were kept in a single collective pen with wooden and metal fences (2.6 m × 4 m). Xylazine (2 mL of Xylased 5%, Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; iv) was used as anaesthetic, given 7–10 min prior to the euthanasia, which was carried out with pentobarbital (2 mL/10 kg Exagon, Richter Pharma, Wels, Austria; iv). Both testes were sampled immediately after euthanasia. Tissues from each testis were fixed in Bouin's fixative solution for 24 h, and the samples were then transferred to 70% ethanol and processed routinely for paraffin embedding. Tissues were further sectioned at 4 µm, stained with periodic acid-Schiff, and counterstained with haematoxylin.

The animals used in this study were part of an unpublished experimental study of bisphenol A reproductive toxicity with permission (no. U34401-3/2015/8) from the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection. All animal procedures were performed in accordance with ethical standards. All procedures involving animals and the experiment complied with the Slovenian Animal Protection Act (Official Gazette of the Republic of Slovenia 38/2013), the Council Directive 2010/63/EU, and ethical principles.

Stages of the seminiferous epithelium cycle

The terminologies used in this study are defined as follows:

- Spermatogenesis: the process in which the primary male germ cells of the seminiferous epithelium proliferate and transform into free spermatozoa (sperm)^{6, 21}
- Spermiogenesis: that part of spermatogenesis when immature round spermatids are transformed into elongated spermatids^{6, 21}
- Cycle of the seminiferous epithelium (hereafter cycle): complete series of successive cellular associations occurring in a particular segment of seminiferous epithelium over time^{6, 21}
- Stages of spermatogenesis: a defined grouping of germ cell types at particular phases of development in cross-sectioned tubules⁸
- Steps of spermiogenesis: defined morphological entities of spermatid development (primarily based on acrosome form and shape and, to a lesser extent, on spermatid head shape and the degree of chromatin condensation)⁸

Ram spermiogenesis was studied using the descriptions proposed by Clermont and Leblond³¹. Stages of the seminiferous epithelial cycle were identified based on the

morphological changes of the spermatids during spermiogenesis, presence of meiotic divisions, and overall associations of the germ cells³². In this study, the seminiferous epithelium cycle in the ram testis was divided into nine distinguishable stages based on criteria suggested for bull³ and rat testes^{6, 33}. However, slight modifications were adopted as Bouin's-fixed, PAS-H-stained sections were used in this study, in contrast to the studies of Berndston and Desjardins³ and Leblond and Clermont⁶, in which Zenker-formol-fixed, periodic acid-Schiff-haematoxylin-stained sections and Orth-fixed, PA-FSA-Harris-haematoxylin-stained, and Flemming-fixed, iron-haematoxylin-stained sections of bull and rat testes were used, respectively. Testes from all six animals were examined to ensure a consistent division of stages. The cross-sections of the seminiferous tubules were examined at 400× and 1,000× magnification.

Results

Cell associations are typical for each stage of development and usually contain spermatogonia, spermatocytes, and spermatids. Light microscopic examination of ram testicular tissue in our study permitted the identification of nine distinguishable stages (I–IV, V, VI, VII, VIII, IX, X, XI, XII) of the cycle. Specific criteria and the types of cells used to identify each stage are described below and presented in Figs. 1 and 2.

Stages I–IV. The basement membrane is lined with spermatogonia. Pachytene spermatocytes are interspersed between spermatogonia and spermatids. Above the pachytene spermatocytes is a layer of round spermatids with occasional visible empty vesicles, but without obvious proacrosomal granules or acrosomal granules visible (round spermatids, steps 1–4). Above and between round spermatids, elongated spermatids are clustered in groups deeply in the cytoplasm of Sertoli cells. Elongated spermatids in step 13 of spermiogenesis are paddle-shaped, oriented towards the basement membrane, and have a large mass of cytoplasm extending into the lumen of the tubule.

Stage V. The basement membrane is lined with spermatogonia. Pachytene spermatocytes are interspersed between spermatogonia and step 5 round spermatids. During stage V, the acrosomal granules of the step 5 spermatids begin to spread over the nuclear membrane, forming a small head cap, covering approximately one-fourth of the nuclear surface; idiosomes near acrosomal granules are only rarely seen. The elongated spermatids in step 13 move further into the tubular lumen.

Stage VI. The basement membrane is lined with spermatogonia. Pachytene spermatocytes are interspersed between spermatogonia and step 6 round spermatids. The acrosomal head cap of step 6 round spermatids cover one-third to one-half of the nuclear surface. The acrosomal head caps are oriented in different directions. Idiosomes migrate distally from the acrosome. Above the step 6 spermatids, the nuclei of step 14 elongated spermatids form a concentric layer lining the outer edge of the tubular lumen. Step

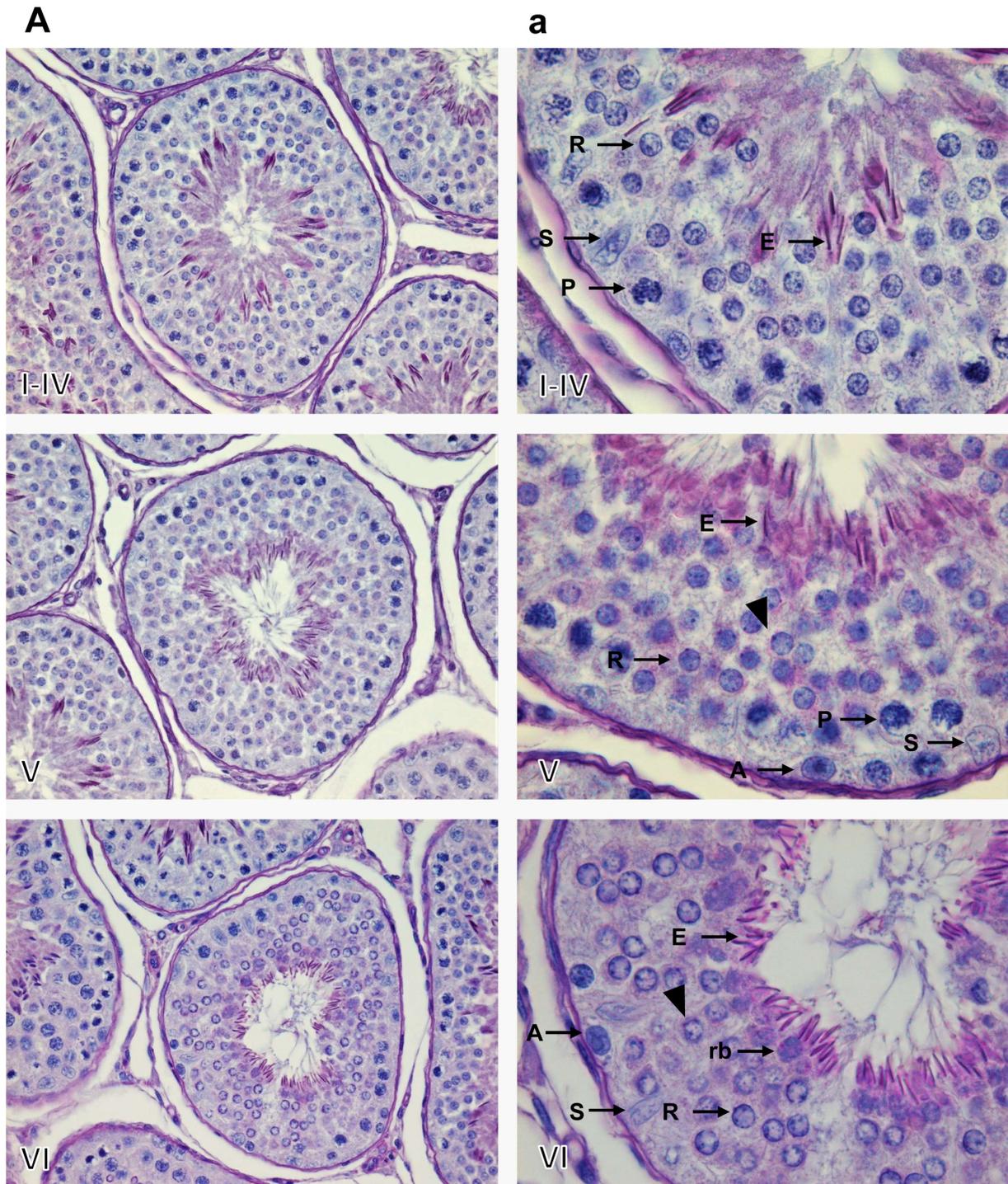


Fig. 1. The 12 stages of the seminiferous cycle in the ram classified based on the development of the acrosomic system. Stages are indicated by Roman numerals (I–XII) and refer to the 12 morphological stages. Pictures on the left side (A, B, C) are taken at 40× magnification; pictures on the right side (a, b, c) are taken at 100× magnification. (A, a) Stages I to VI represent the early stages showing two generations of spermatids, round and elongated. Stages I–IV: presence of round spermatids and step 13 spermatids. Stage V: presence of step 5 round spermatids with a small acrosomal head cap, step 13 elongated spermatids. Stage VI: presence of step 6 round spermatids with an acrosomal head cap covering one-fourth to one-half of the nuclear surface, palisading elongated step 14 spermatids and abundant residual bodies. Legend: S = Sertoli cells, A = A spermatogonia, R = round spermatids, E = elongated spermatids, P = pachytene spermatocytes, rb = residual bodies. Acrosomes are marked with black arrowheads in stage V and stage VI

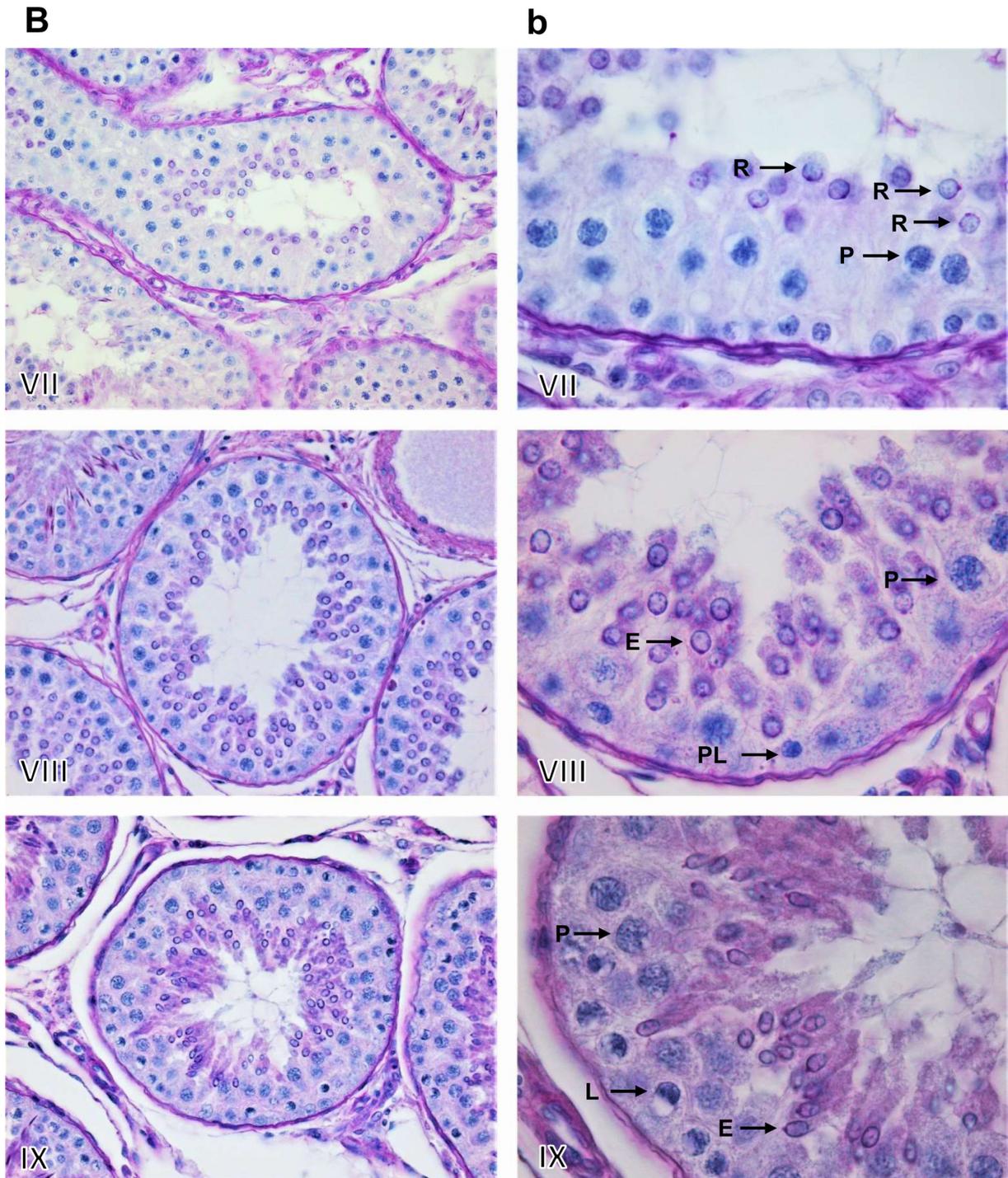


Fig. 1. Continued. (B, b) Stages VII to IX represent the late stages showing one generation of spermatids. Stage VII: presence of step 7 round spermatids with an acrosomal head cap covering one-half of the nuclear surface. Stage VIII: presence of step 8 elongated spermatids with an acrosomal granule oriented towards a basal membrane. Stage IX: presence of step 9 spermatids with elongating nuclei and protruding acrosome. Legend: R = round spermatids, E = elongated spermatids, P = pachytene spermatocytes, PL = preleptotene spermatocytes, L = leptotene spermatocytes.

14 spermatids have proximal protoplasmic droplets. In addition, residual bodies are observed at the concentric layer.

Stage VII. Spermatogonia line the basement membrane, and pachytene spermatocytes are interspersed be-

tween spermatogonia and step 7 round spermatids. Only step 7 spermatids are present at this stage as elongated spermatids were released from the seminiferous epithelium in the previous stage. The acrosomal head caps of step 7 round

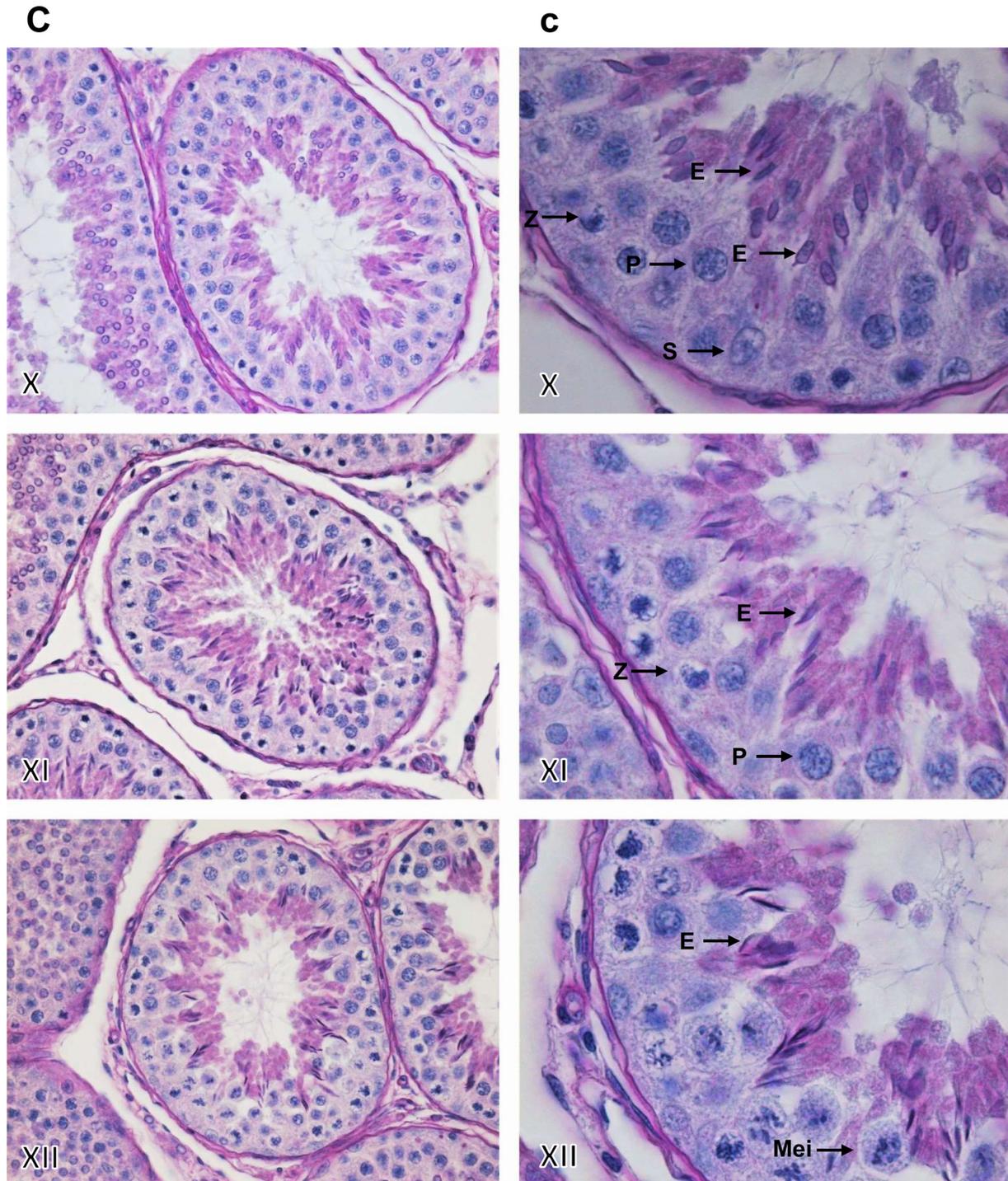


Fig. 1. Continued. (C, c) Stages X to XII represent the late stages showing one generation of spermatids. Stage X: presence of step 10 elongated spermatids. Acrosomes and nuclei are further elongated. The nuclei are also further flattened; hence, step 10 spermatids can be seen in two forms, one being narrower (lateral profile) than the other. Stage XI: presence of step 11 elongated spermatids with paddle-shaped nuclei with pointed triangular acrosome. Stage XII: presence of step 12 elongated spermatids with paddle-shaped nucleus and meiotic figures. Legend: E = elongated spermatids, P = pachytene spermatocytes, Z = zygotene spermatocytes, Mei = meiotic division.

spermatids cover half of the nuclear surface and are still oriented in different directions. Idiosomes are rarely observed at the distal edge of the head cap.

Stage VIII. The basement membrane is lined with sper-

matogonia; above spermatogonia, there are scattered preleptotene spermatocytes, which are situated near the basement membrane and have round nuclei and fine granular chromatin. On top of them, there is a layer of pachytene spermatoc-

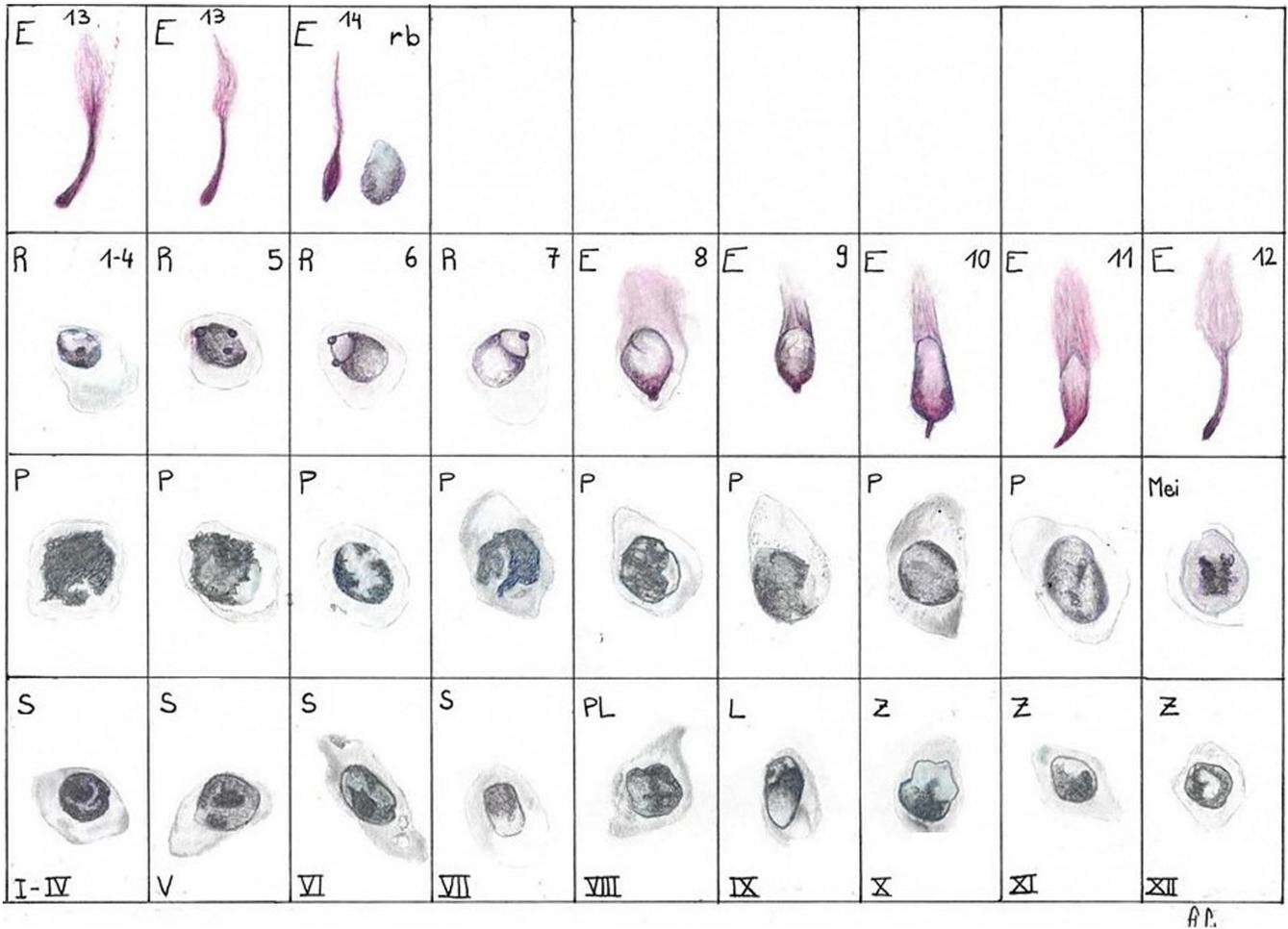


Fig. 2. The Roman numerals I–XII indicate the stage(s) of each separate column, and each column depicts cellular associations at specific stage of the cycle of the seminiferous epithelium in ram testis

The types of germ cells observed were as follows: S, spermatogonia; PL, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; and Mei, meiotic division of spermatocytes.

Round and elongated spermatids are illustrated in the upper two rows: R, round spermatids; E, elongated spermatids; and rb, residual bodies.

cytes and step 8 spermatids, whose nuclei are roundish to slightly ovoid with a large cytoplasmic tail. The acrosomal granules and head caps of step 8 spermatids are almost all oriented towards the basement membrane.

Stage IX. The basement membrane is lined with spermatogonia and topped with leptotene spermatocytes and pachytene spermatocytes. A dense network of thin chromosomal filaments in the nuclei determines the leptotene spermatocytes. Step 9 spermatid nuclei are more elongated than that in stage VIII; step 9 spermatid acrosomes are enlarged and protruded approximately half the size between step 8 and step 10 spermatids. Step 9 spermatids have a long, wide cytoplasmic tail.

Stage X. The basement membrane is lined with spermatogonia. Above spermatogonia, there are zygotene and pachytene spermatocytes. Zygotene spermatocyte chromatin filaments are paired and often appear displaced within

the nucleus. Acrosomes of step 10 spermatids are elongated for one-third of the nucleus, and step 10 spermatid nuclei are even further elongated. Step 10 spermatids can be seen in two forms, some being narrower (lateral profile) than others.

Stage XI. The basement membrane is lined with spermatogonia with zygotene and pachytene spermatocytes above. Elongated spermatids can be seen in two ways: the lateral profile of step 11 spermatids is somewhat curved and pointed at the end, whereas the frontal profile is wide and the apex becomes clearly pointed.

Stage XII. The basement membrane is lined with spermatogonia with zygotene and pachytene spermatocytes above. Spermatocyte meiosis is a characteristic of this stage and is the initial process for step 1 spermatid formation. Some elongated step 12 spermatids are deep between the two layers of meiotic cells; however, most of them are on the top. The acrosome becomes bluntly pointed.

Discussion

According to Berndston and Desjardins³, morphological changes in the acrosomic system and in the nuclei of developing spermatids were evaluated as a basis for classifying the stages of the seminiferous epithelium cycle in the bovine testis. In their study, Clermont and Leblond³¹ stated that the development of the acrosomic system is almost the same in the bull as that in the ram. Hence, in our study, we attempted to use the staging scheme for bovine testes to stage ram testes and determine whether it is suitable for that purpose. However, we observed a number of differences, which are discussed below, mainly in the shape of the developing spermatids in rams in comparison with those in bulls. For detailed illustrations of the morphology of developing spermatids in rams, we also studied Clermont and Leblond's³¹ publication, where the morphology of the ram developing spermatid is represented precisely, and our observations largely concur with their illustrations of step 5 to step 15 spermatids.

In the seminiferous epithelium in the bovine testis, a step 7 spermatid in stage VII is presented with the head cap, and the acrosomal granules are oriented towards the lumen³. In our study, the head cap and acrosomal granules of step 7 spermatids were oriented in multiple directions.

When applied to both species, the nuclei of step 9 spermatids in stage IX do not appear flattened when viewed laterally. In contrast, step 9 spermatid nuclei in the bovine testis are more triangular, and the acrosome is not more elongated than that in step 8 spermatids, whereas nuclei of step 9 spermatids in our sections, when viewed frontally, are more ovoid shaped as that in step 8 but more elongated and with elongated acrosome.

Bovine step 10 spermatids in stage X had slightly shorter acrosomes³ than those in our study.

According to Berndston and Desjardins³, the main feature of stage XI is the absence of an elongated acrosomal protrusion associated with spermatids in the preceding stage. The acrosome and head cap of bovine step 11 spermatids become indistinguishable and form a broad crescent- or wedge-shaped structure protruding from the apex of the nucleus. We observed the same fusion in ram step 11 spermatids; however, the shape of the merged acrosome and head cap structure is triangular with the base of the triangle blending into the head cap in the frontal view. In the lateral view, step 11 spermatids were slightly rounded.

In Berndston and Desjardins³ study, it was possible to categorise 14 steps of spermatid development. The first 12 steps were used as criteria to identify each stage of the seminiferous epithelium cycle and its specific cell types. We were unable to differentiate steps 1 to 4, as described in their publication, because we could not detect Golgi zones, proacrosomal granules, or acrosomal granules, which are the hallmarks of stages I, II, III, and IV. The reason was deemed to be that Bouin's fixative was used in our study.

Furthermore, other causes, such as fixation artefact, cannot be excluded. Therefore, in our study, we identified 11 recognisable steps involved in spermatid development, and nine of these steps were used as criteria to identify each stage of the seminiferous epithelium cycle. It was decided to merge stages I, II, III, and IV into a group stage labelled as I–IV. Stages I–IV are followed by stage V to stage XII. The reason for such labelling is to keep the staging classification aligned with the regular scheme of seminiferous epithelium staging in bovine testis³. However, based on the studies by Clermont and Leblond¹² on ram spermiogenesis and their precise description of 15 steps of spermiogenesis in rams and Berndston and Desjardins³ of the bovine spermatogenesis cycle, we believe that the ram seminiferous epithelium cycle can be further divided into at least 12 distinct stages based on acrosome development.

In contrast to the previously discussed Zenker-formol³ or Orth fixation³¹, Bouin's fixation was used in our study. For an in-depth study of the seminiferous cycle of rams, especially with regard to stages I–IV, and the occurrence of different types of spermatogonia, one of the latter fixatives would be optimally used in future fundamental studies. For regular testicular toxicity testing, however, standard Bouin's fixation or Davidson fluid fixation is recommended³⁴. Therefore, the ability to perform seminiferous cycle staging in rams on Bouin's-fixed tissue indicates the importance of our study.

We conclude that the staging described for bovine testes³ is suitable for staging seminiferous epithelium in ram testes. However, the schematic illustration of the morphology of the developing bovine spermatids in the above-mentioned publication differs slightly from ours. When familiarisation with seminiferous epithelium cycle staging in ram is required, schematic illustrations by Berndston and Desjardins³ in addition to photomicrographs and a scheme from this study and specific illustrations of developing spermatids by Clermont and Leblond³¹ together can greatly facilitate the recognition of a specific stage and the consequential abnormalities.

In the present study, seminiferous epithelium cycle staging based on acrosome development in ram testes was proposed. A description of the identification criteria for the nine stages of the cycle, illustrative microphotographs, and a scheme are given for PAS-H-stained, Bouin's-fixed sections.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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