Diurnal Changes in Murine Spermatogenesis

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We analysed testicular samples of NMRI mice every 2 h of a day in order to determine whether there is a circadian rhythm in spermatogenetic activity. We used flow cytometry after staining the DNA with DAPI. The highest proportion of DNA synthesizing cells (mainly spermatogonia and preleptotene spermatocytes) was seen at 8 p.m. and especially at 10 p.m.; the lowest proportion was observed at 2 p.m. At 6 a.m., the percentage of round spermatids increased significantly, whereas the fraction of 4c-cells decreased at that time. Our results of a diurnal rhythm of spermatogenic DNA synthesis rate are in contrast to another publication of other authors (Oakberg and Crosswait, 1983).

Introduction

Since Bergonié's and Tribondeau's work (1906) it is known that cell kinetics influence the sensitivity of tissues to physical and chemical noxae. Especially circadian rhythms must be taken into account, not only for medical applications (Møller *et al.*, 1980; Hrushesky and Bjarnason, 1993), but also if proliferative activities, *e.g.* of spermatogenic cells, shall serve as a measure of environmental noxae (Otto and Oldiges, 1986). Here, the proliferative activity of spermatogenic cells in mice kept under natural light conditions is investigated by DNA analysis using flow cytometry.

Materials and Methods

Male NMRI mice (*Mus musculus*, Institut für Versuchstierzucht, Hannover) aged 8 weeks were used. The experiment was performed at the 15th and 16th of August 1983 at natural conditions. Sunrise and sunset took place at 6.07 h a.m. and 8.44 h p.m. summertime (MEZ minus 1 h). That is a long photoperiod of about 14.5 h. Every 2 h five mice were killed during 24 h.

The testes were minced with surgical blades after removal of the tunica albugenia, fat, epididymis and connective tissue in preparation for flow cytometry. In contrast to microscopy, flow cytometry does not allow to visualize the spatial distribution of the cell associations typical for different stages (*cf.* Fig. 1 a, b) (Oakberg, 1956) since the cells are measured in a solution. However, quantification of the different testicular cell types is more easy and much faster by flow cytometry. Sample preparation and staining procedure with the DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole · 2 HCl (DAPI) has been described before (protocol 1, *cf.* Otto and Oldiges, 1986), as well as the DNA histogram of testicular cells as measured by flow cytometry and verified by cell sorting (Hacker-Klom *et al.*, 1985, 1989; Tatchen *et al.*, 1989). Basically, six groups of spermatogenic cells may be differentiated (Fig. 1 b): 1. haploid elongated spermatids (1 cc), 2. haploid round spermatids (1 c), 3. diploid (2 c) cells (Leydig cells, epithelial cells, spermatogonia in the G 1-phase of the cell cycle, and preleptotene spermatocytes), 4. cells synthesizing DNA (mainly spermatogonia and spermatocytes), 5. 4 c-cells, mainly primary spermatocytes in pachytene, and 6. diploid elongated spermatids (Hacker-Klom *et al.*, 1989). The calculation of the different testicular cell types was performed using the cumulative frequency distribution (Meier, 1983). The coefficients of variation allowing to determine the measuring accuracy were about 2% for peak III in each DNA histogram. Each testis was analysed separately. The arithmetic means and standard deviations were calculated for each of the six cellular populations mentioned above. The significance at the 0.05 level

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of the differences between the arithmetic means of the groups was tested by the Student t-test. A particle analysis system PAS II (Partec AG, Münster) was used for the flow cytometric analysis.

Results

At 6 o'clock a.m., the percentage of round spermatids has increased significantly over the arithmetic mean ±95% confidence limits of the combined time points (Fig. 2a). There were no significant changes at any time of the percentages of the 2c-cells and the haploid elongated sperma-

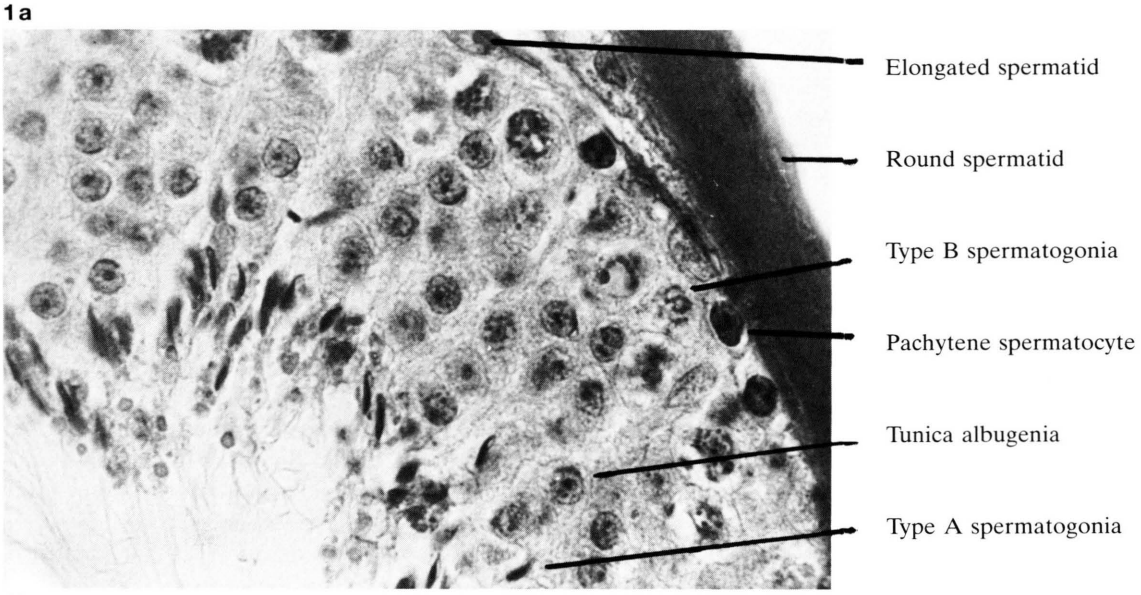

Fig. 1. a) Microphotograph (×928) of a cross section through a murine testicular tubule showing a stage VI (6) after staining with PAS/hematoxylin. The different cellular components and the tunica albugenia are indicated in the photograph. b) Upper panel: DNA histogram of the testicular cells of a mouse. *x*-Axis: relative DNA content per cell; *y*-axis: number of cells per channel. Peak I represents haploid elongated spermatids (named 1cc since these cells do not stain proportional to their DNA content because of their highly condensed chromatin), peak II haploid round spermatids (1c), peak III 2c-cells, and peak IV 4c-cells. Between peaks III and IV, cells synthesizing DNA are registered. Lower panel: composition of the DNA histogram of the upper panel. R!, 1st and 2nd meiotic division; S, DNA synthesis phase of cell cycle; M, mitosis.

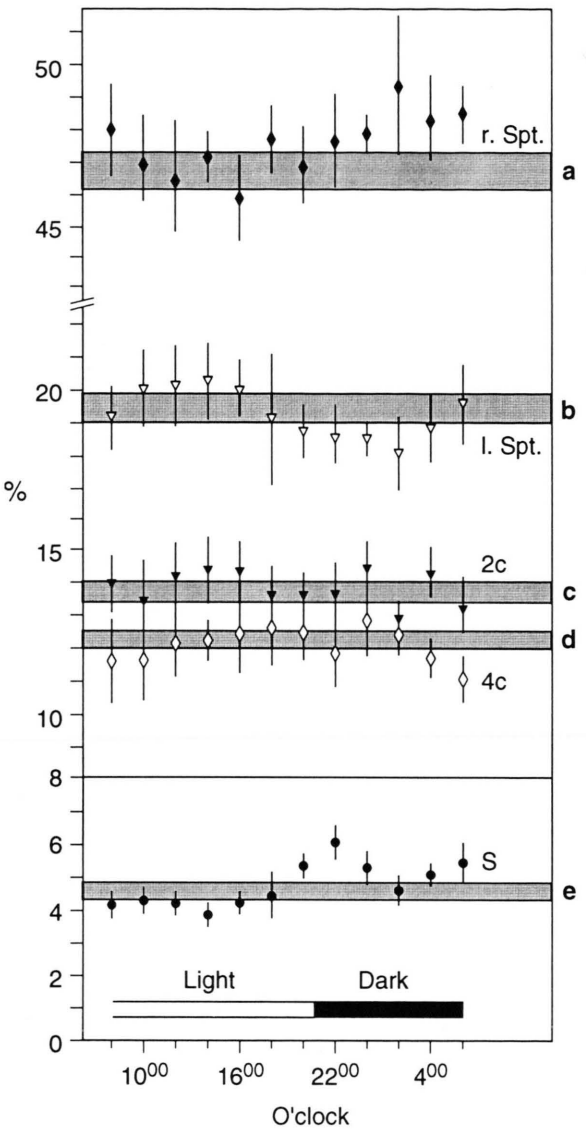

Fig. 2. Changes in the percentages of the a) haploid round spermatids (r. Spt.), b) haploid elongated spermatids (l. Spt.), c) 2c-cells (2c), d) 4c-cells (4c), and e) cells synthesizing DNA (S) in dependence of the time of the day (MEZ minus 1 h = summertime).

tids (Fig. 2b, c). The percentage of 4c-cells is reduced significantly at 6 o'clock a.m. (Fig. 2d).

The arithmetic mean ±95% confidence limits of the percentage of DNA synthesizing cells of the 60 mice analyzed was 4.6 ± 0.2% (Fig. 2e). At 2 o'clock p.m., there were significantly fewer cells synthesizing DNA than the arithmetic mean ±95% confidence intervals of all mice. At 8 p.m. and at 10 o'clock p.m., the percentage of cells synthesizing DNA was increased significantly. The highest value was reached at 10 o'clock p.m. with an arithmetic mean of 6% of all testicular cells synthesizing DNA. At 6 a.m., there is a non-significant increase of the percentage of cells synthesizing DNA. Between 8 a.m. and 12 a.m., and between 4 p.m. and 6 p.m., there are no significant changes in the percentages of DNA synthesizing spermatogenic cells.

Discussion

The fraction of 4c-cells (mainly spermatocytes) is decreased and the fraction of round spermatids is increased significantly above the arithmetic mean ±95% confidence intervals of all values at 6 a.m. We conclude that meiotic divisions of spermatocytes occur preferentially at that time of the day shortly after sunrise. In the hibernating snail *(Helix aspersal),* too, the photoperiod has an important effect on the second meiotic division being probably most sensitive to environmental factors (Medina *et al.,* 1988). Since no circadian periodicity in spermatogonial apoptosis was detected in the rat (Allan *et al.,* 1992), we do not expect a diurnal rhythm of programmed cell death in murine spermatocytes either.

Oakberg and Crosswait (1983) were not able to detect a circadian rhythm in the kinetics of spermatogenesis in hybrid mice. Here, however, a significant change in the DNA synthesis rate of spermatogenesis depending on the time of the day is described. Possibly, the discrepancy between their and our results is a consequence of the different methods used: Oakberg and Crosswait used autoradiography which only allows the analysis of small numbers of cells. We, however, tested by flow cytometry about 10,000 cells per testis of each of the 60 mice of the experiment, that are 1,200,000 cells altogether.

Circadian rhythms in cell proliferation of rodent epithelia were described by Møller *et al.* (1980) in the hamster cheek pouch. Between noon and 4 p.m., DNA synthesis rate is lowest in hamster cheek pouch and in testicular cells as shown here. The peak of DNA synthesis, however, is at 2 a.m. in hamster cheek pouch, but between 8 p.m. and 10 p.m. in the testicular cells of mice. Thus, circadian rhythms are likely to depend on species and

type of tissue. The results of this investigation show that noxious influences on cell proliferation can properly be assessed only if cell kinetics are known and taken into account in relation to the time of sampling.

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