Partial synchronization of spermatogenesis in the immature Djungarian hamster, but not in the immature Wistar rat

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The frequencies of the cellular associations of the seminiferous epithelium were determined at various ages after birth in immature Djungarian hamsters and Wistar rats. The frequencies of the cellular associations present in immature animals were then compared with the frequencies of the corresponding pooled stages in adult animals. At 15 days of age, the cellular associations present in Djungarian hamsters could be divided into four groups based on the presence of A_3 , intermediate (In), or B (B_1 and B_2) spermatogonia, or preleptotene–leptotene–zygotene spermatocytes. Compared with adult animals, the group containing spermatocytes was found to be enriched by 52%, while the frequency of the B spermatogonia was reduced by 55%. At 22 days of age, the cellular associations of spermatogenesis were classified into five groups: pachytene spermatocytes associated with A_3 spermatogonia, In spermatogonia, B spermatogonia, or preleptotene–leptotene–zygotene spermatocytes, or the meiotic divisions. The stages containing preleptotene–leptotene–zygotene spermatocytes and those containing A_3 spermatogonia were enriched by 35% and 59% compared with adult values, respectively. The meiotic divisions were enriched by 21%, but this increase was not significant. The frequency of the B spermatogonia was reduced by 62%. In addition, the synchronization factor, calculated with the computer program SYNTTEST, was increased to 1.48 ± 0.05 (mean $\pm$ SEM) and 1.38 ± 0.03 at 15 and 22 days of age, respectively, and was significantly higher ($P < 0.001$ at both ages) than the adult value of 1.08 ± 0.02 . However, after 22 days of age the synchronization factor did not differ from that in adult animals. These data indicate that spermatogenesis was partially synchronized in Djungarian hamsters up to 22 days of age. In Wistar rats from 22 days of age onwards, all tubular cross-sections could be classified. No consistent enrichment of epithelial stages was found at any age examined. Furthermore, at any age tested the synchronization factor did not differ from adult values, confirming the lack of synchronization in the immature Wistar rat. The partial synchronization in immature Djungarian hamsters probably originates from the rather synchronous start of gonocyte proliferation. The lack of synchronization in immature rats is probably a consequence of the slower start of gonocyte proliferation in this species.

Introduction

The stages of the cycle of the seminiferous epithelium in tubular cross-sections of adult animals show a random distribution. The frequency of the spermatogenic stages is therefore regarded as an adequate measure of their relative duration (Leblond and Clermont, 1952a). The duration of the stages is not affected by alterations in gonadotrophin concentrations caused by hypophysectomy, hypophysectomy and concomitant treatment with testosterone propionate or chorionic gonadotrophin (Clermont and Harvey, 1965), or hemicastration (Noller *et al.*, 1977). Depletion of specific spermatogenic cell types by exposure to 43°C for 15 min (Chowdhury and

Steinberger, 1964), or by treatment with alkylating agents (De Rooij and Kramer, 1970) does not affect the duration of the remaining stages. In the immature mouse the frequencies of the stages were shown to be altered by Nebel *et al.* (1961), whereas Kluin *et al.* (1982) found that they were similar to those in adult mice.

When vitamin A is administered to vitamin-A-deficient rats, a labelling index of type A spermatogonia of about 50% is found after 24–30 h (Van Pelt and De Rooij, 1990), and subsequently a highly synchronous development of spermatogenesis takes place (Morales and Griswold, 1987; Van Beek and Meistrich, 1990; Van Pelt and De Rooij, 1990). In immature Djungarian hamsters spermatogenesis starts rather synchronously, with a labelling index of gonocytes of nearly 50% (Van Haaster *et al.*, 1993). We investigated whether this high rate of gonocyte proliferation also results in a synchronous

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development of spermatogenesis. In addition, since testicular cells of immature rats are frequently used for studies *in vitro*, we investigated whether synchronous development of spermatogenesis occurs in immature rats.

Materials and Methods

Animals

Djungarian hamsters (*Phodopus sungorus sungorus*) were born in our breeding colony, which was generously supplied by P. Niklowitz (Max Planck Clinical Research Unit for Reproductive Medicine, Münster). The colony was checked for litters once a day. Djungarian hamsters were killed, by decapitation or chloroform inhalation, on consecutive days from 4 to 9 days of age and at 15, 22, 29, 43 and 64 days of age. The 4–9-day-old groups each consisted of six or seven animals; the 15–64-day-old groups consisted of five or seven animals. At all ages the animals were derived from at least two different litters.

Wistar rats at known stages of pregnancy were obtained from Harlan CPB (Zeist) or the Central Animal Facilities of the University of Utrecht (Utrecht). The rats were checked for litters twice daily. The rats were killed, by decapitation or chloroform inhalation, at consecutive days from 3 to 8 days of age and at 15, 22, 28, 43, 64 and 85 days of age. Each age group consisted of four animals from at least two different litters.

For both species the day of birth was designated as day 1, and pups were weaned at 3 weeks of age.

Histology

Two hours before they were killed the 4–9-day-old hamsters and the 3–8-day-old rats were injected s.c. with 150 mg bromodeoxyuridine (BrdU) kg^{-1} body mass (Sigma Chemical Co, St Louis, MO). The testes of these animals were fixed in Carnoy's fluid. The testes of the animals from 15 days of age onwards were fixed in Bouin's fluid. All testes were embedded in glycol methacrylate:butanediolmonoacrylate (BDH Laboratory Supplies, Poole; Klinipath BV, Zevenaar) at a ratio of 85:15. The BrdU label was visualized in 3 μm sections of the testes fixed in Carnoy's fluid using monoclonal anti-BrdU and peroxidase-conjugated rabbit anti-mouse antibodies followed by a diaminobenzidine- H_2O_2 reaction, according to Harms *et al.* (1986) and Van de Kant *et al.* (1990). The sections were counterstained with Gill's haematoxylin (Polysciences Inc, Warrington, PA). In these sections the BrdU labelling index of gonocytes was scored. Sections, 5 μm thick, of the testes fixed in Bouin's fluid were made at intervals of 250 μm , and stained with the periodic acid Schiff (PAS) technique and counterstained with Gill's haematoxylin. In these sections the frequencies of the spermatogenic cell types were scored.

Morphological analysis

Gonocytes were identified using the criteria described by Kluin and De Rooij (1981). The labelling index of gonocytes was determined by counting at least 500 gonocytes per animal.

The morphological criteria used to identify the spermatogenic cell types and the position of the mitoses of the differentiating spermatogonia in the spermatogenic cycle of Djungarian hamsters have been described by Van Haaster and De Rooij (1993a). A_3 , intermediate (In) and B spermatogonia could be identified with certainty. However, B_1 and B_2 spermatogonia could not be distinguished from each other. As the transition between the steps of spermatocyte development is gradual, a clear identification of these cell types is sometimes difficult. The frequencies of the preleptotene, leptotene and zygotene spermatocytes were therefore pooled. However, the pachytene spermatocytes could be distinguished separately.

The differentiating spermatogonia (A_4 , In and B spermatogonia could be distinguished) in Wistar rats were identified according to Hilscher *et al.* (1969). The position of mitoses of the differentiating spermatogonia during the seminiferous cycle was found to be as described by Hilscher *et al.* (1969). The transition between the zygotene and pachytene spermatocyte stages was found to occur between stages XIII and XIV, as described by Hess (1990). To distinguish between the 14 stages of spermatogenesis in Wistar rats the criteria of Leblond and Clermont (1952b) and those of Hess (1990), who described criteria for identifying the stages in plastic-embedded sections, were used.

Frequencies of the stages

The frequencies of the cellular associations present in immature animals were determined by examining at least 200 tubular cross-sections per animal. When a transition occurred from one cellular association to a subsequent one in a tubular cross-section, that association occupying more than 50% of the tubular cross-section was scored.

The data on the frequencies of the stages of the seminiferous cycle in adult Djungarian hamsters and Wistar rats were derived from our previous studies (Van Haaster and De Rooij, 1993a, b). In the study of the Djungarian hamster seminiferous epithelium, at least 200 tubular cross-sections per animal, in 17 animals (5–10 months of age), were staged. In the study dealing with spermatogenesis in rats at least 200 tubular cross-sections per animal, in 12 animals (6 months of age), were staged.

To compare the frequencies of the cellular associations in immature animals with those in adult animals, the frequencies of the stages in adult animals were pooled according to the position of mitoses of the differentiating spermatogonia and the spermatocyte steps during the seminiferous cycle.

Synchronization factor

For each animal a synchronization factor was calculated with the computer program SYNTTEST, developed by Van Beek and Meistrich (1990). This program first calculates the stage with the lowest relative occurrence; this point is identified as the 0/100 percentile point. Starting with this point, the 15.87, 50 and 84.13 percentile points are calculated. In normally distributed data these points would indicate the mean $\pm$ SD. The window width is defined as the part of the cycle (expressed as a percentage) containing 68.26% of the staged

Table 1. The frequencies of the stages of the seminiferous cycle in immature and adult Djungarian hamsters

Stage	Day 15 (7) ^a	Day 22 (5)	Day 29 (7)	Day 43 (7)	Day 64 (5)	Adult (17)
I } II }	12.9 ± 1.1 ^b [12.7 ± 0.7]	20.2 ± 1.5^{b***} [12.7 ± 0.7]	8.7 ± 0.7	8.0 ± 0.9	9.0 ± 1.2	7.7 ± 0.6
III } IV }	9.2 ± 1.8 ^c [12.0 ± 0.7]	12.6 ± 1.4 ^c [12.0 ± 0.7]	6.5 ± 0.4*	4.4 ± 0.6	6.9 ± 0.4*	4.2 ± 0.4
V } VI }	15.4 ± 1.4 ^{d***} [34.3 ± 1.0]	12.9 ± 1.3 ^{d***} [34.3 ± 1.0]	28.5 ± 1.4 ^d [34.3 ± 1.0]	4.7 ± 0.8	5.6 ± 0.3	7.4 ± 0.6
VII } VIII }				13.3 ± 1.0	16.3 ± 2.2	15.3 ± 0.8
IX } X }	62.5 ± 1.6^{e***} [41.0 ± 1.3]	46.0 ± 1.4^{e***} [34.1 ± 1.1]	33.6 ± 2.0 ^e [34.1 ± 1.1]	20.0 ± 1.4	22.9 ± 1.3	23.3 ± 1.2
XI } XII }				6.0 ± 0.7	5.1 ± 0.7	6.7 ± 0.5
				7.8 ± 0.9*	5.6 ± 0.3	4.6 ± 0.4
				7.6 ± 0.4**	6.7 ± 0.4	4.5 ± 0.4
				9.0 ± 1.8	5.0 ± 0.6	6.7 ± 0.6
		8.2 ± 0.8 ^f	6.7 ± 1.0 ^f	6.3 ± 0.4	5.6 ± 0.3	6.8 ± 0.5

Data are expressed as means ± SEM. The frequencies of the corresponding pooled stages in adult animals are given in brackets. The frequencies that are printed in bold are significantly greater than those of adult animals.

^aThe number of animals tested is given in parentheses.

When necessary, the stages were pooled, based on the presence of ^bA₃ spermatogonia, ^cintermediate (In) spermatogonia, ^dB₁ and B₂ spermatogonia, or ^epreleptotene–leptotene–zygotene spermatocytes. ^fThe stage at which meiotic divisions occur is distinguished as a separate group at the ages tested.

P* < 0.01 and **P* < 0.001 compared with adult values.

tubular cross-sections, distributed equally on both sides of the 50 percentile point. The synchronization factor is the window width of control animals divided by the window width of the synchronized animals (Van Beek and Meistrich, 1990). Control data for the SYNTTEST program consist of the total number of tubules found in each stage in the control animals. When spermatogenesis is synchronized in an experimental animal, the synchronization factor is greater than 1.

For each immature animal, a synchronization factor relative to the whole group of adult animals was calculated. To compare the immature animals with the adult animals, we also calculated a synchronization factor for each adult animal relative to the whole group of adult animals.

Statistical analysis

To obtain a normal distribution of the data, the frequencies of the cellular associations were first divided by 100 to obtain a fraction, which was then transformed to an arcsine. Subsequently, data were analysed using Student's *t* test modified according to Bonferroni. Differences were considered significant when *P* < 0.01. The synchronization factors were analysed with Bonferroni's *t* test under the assumption of normality. Data are expressed as the mean ± SEM.

Results

Frequencies of the stages in immature animals

In immature Djungarian hamsters at 15 days of age, the seminiferous tubules contained stages ranging from A₃ spermatogonia to pachytene spermatocytes associated with preleptotene spermatocytes. The cellular associations present could be divided into four groups, based on the presence of A₃, In or B (B₁ and B₂) spermatogonia, or preleptotene–leptotene–zygotene spermatocytes. When the frequencies of these cellular associations were compared with the frequencies of the corresponding pooled stages in adult animals, the group containing spermatocytes (i.e. from the middle of stage VII to stage XII) was found to be enriched by 52% above adult values (*P* < 0.001). Concomitantly, the occurrence of the B spermatogonia (i.e. from stage V to the middle of stage VII) was reduced by 55% compared with adult values (*P* < 0.001) (Table 1).

At 22 days of age in the Djungarian hamster the tubular cross-sections contained at least pachytene spermatocytes associated with A₃ spermatogonia up to round spermatids in stage VI. The cellular associations could be divided into five groups, based on the presence of pachytene spermatocytes

Table 2. The frequencies of the stages of the seminiferous cycle in immature and adult Wistar rats

Stage	Day 22 (4) ^a	Day 28 (4)	Day 43 (4)	Day 64 (4)	Day 85 (4)	Adult (12)
I } II } III } IV } V } VI } VII } VIII } IX } X } XI } XII } XIII } XIV }	14.7 ± 2.0 ^b [17.9 ± 1.0]	19.4 ± 2.3 ^b [17.9 ± 1.0]	14.9 ± 1.3	14.2 ± 1.8	11.5 ± 1.3	15.6 ± 0.9
	9.5 ± 1.2 ^c [12.9 ± 1.0]	10.4 ± 1.5 ^c [12.9 ± 1.0]	4.6 ± 0.4	5.6 ± 1.1	5.3 ± 1.1	4.2 ± 0.7
			2.8 ± 0.7	2.7 ± 0.8	4.6 ± 1.0	4.1 ± 0.4
			3.0 ± 0.4	3.2 ± 1.0	4.0 ± 0.4	4.1 ± 0.5
	11.7 ± 1.4 ^d [9.8 ± 0.7]	8.9 ± 1.9 ^d [9.8 ± 0.7]	6.3 ± 1.3*	5.7 ± 1.0	4.4 ± 1.0	3.5 ± 0.3
			7.3 ± 0.5	8.8 ± 0.7	9.0 ± 0.3	6.3 ± 0.8
			20.3 ± 1.9	24.2 ± 1.5	21.2 ± 1.9	23.8 ± 1.0
			5.9 ± 0.4	8.3 ± 1.6	6.3 ± 1.2	5.0 ± 0.6
			5.0 ± 1.0	3.6 ± 1.1	4.6 ± 0.6	5.8 ± 0.5
	64.1 ± 2.0 ^e [59.4 ± 1.6]	61.3 ± 4.1 ^e [59.4 ± 1.6]	3.0 ± 0.3	2.0 ± 0.5	3.3 ± 0.6	3.3 ± 0.2
			3.5 ± 0.4	3.6 ± 0.5	4.1 ± 0.3	3.1 ± 0.3
			3.6 ± 0.5	3.8 ± 0.5	5.7 ± 1.3	5.9 ± 0.8
			9.6 ± 1.1	9.5 ± 1.8	11.5 ± 1.7	10.4 ± 0.8
			10.2 ± 1.6**	4.8 ± 0.4	4.6 ± 0.7	5.0 ± 0.4

Data are expressed as means ± SEM. The frequencies of the corresponding pooled stages in adult animals are given in brackets. The frequencies that are printed in bold are significantly increased.

^aThe number of animals tested is given in parentheses.

When necessary, the stages were pooled, based on the presence of pachytene spermatocytes associated with ^bA spermatogonia, with ^cintermediate (In) spermatogonia, or with ^dB spermatogonia, or ^epreleptotene–leptotene–zygotene spermatocytes.

* $P < 0.01$ and ** $P < 0.001$ compared with adult values.

associated with A₃ spermatogonia, with In spermatogonia, with B spermatogonia, or with preleptotene–leptotene–zygotene spermatocytes, or the meiotic divisions. An enrichment was found of cellular associations containing pachytene spermatocytes associated with A₃ spermatogonia, and of those containing pachytene spermatocytes with preleptotene–leptotene–zygotene spermatocytes (i.e. from the middle of stage VII to stage XI; $P < 0.001$ for both groups). The crude counts showed that the enrichment observed in the pachytene spermatocytes with A₃ spermatogonia was mainly due to an increased occurrence of tubular cross-sections containing round spermatids in stages I and II. The frequency of the meiotic divisions (stage XII) was also increased at this age, but this increase was not statistically significant. A reduced frequency was found for pachytene spermatocytes associated with B spermatogonia ($P < 0.001$).

At 29 days the cellular associations found ranged from pachytene spermatocytes with B spermatogonia up to elongated spermatids in stage VII. These spermatogenic cell types were classified into seven groups: pachytene spermatocytes associated with B spermatogonia and with preleptotene–leptotene–zygotene spermatocytes, the meiotic divisions and

stages I–IV. An enrichment was found in stages II and III compared with adult values, mainly due to an increased frequency of tubular cross-sections in these stages containing elongated spermatids ($P < 0.01$ for both stages). From 43 days of age onwards, all tubules contained round or elongated spermatids and were classified into the 12 stages of spermatogenesis. At 43 days of age stages IX ($P < 0.01$) and X ($P < 0.001$) were enriched. At 64 days only stage III was found to be enriched ($P < 0.01$).

At 15 days of age in the Wistar rat, some tubules could still be found that contained only unidentifiable type A spermatogonia, which could either be undifferentiated or early differentiating type A (A₁–A₃) spermatogonia. Hence, no reliable frequency distribution could be made at 15 days of age. However, at 22 days of age the cell types present ranged from preleptotene spermatocytes up to round spermatids in stage III. They were assigned to the following groups: preleptotene–leptotene–zygotene spermatocytes, pachytene spermatocytes associated with A, with In, or with B spermatogonia. No differences were found when the frequencies were compared with those in adult animals (Table 2). At 28 days, spermatogenesis had advanced from

Fig. 1. The synchronization factor of the seminiferous epithelium of (a) Djungarian hamsters (○) and (b) Wistar rats (●) during development. Vertical bars represent SEM. * $P < 0.001$ compared with adult animals.

at least pachytene spermatocytes with A spermatogonia up to round spermatids in stage VII. No significant differences were found when the frequencies were compared with those in adult animals. At 43 days of age, when spermatogenesis could be divided into 14 stages, stages V ($P < 0.01$) and XIV ($P < 0.001$) were found to be enriched. However, at 64 and 85 days, no significant differences were observed.

Synchronization factor

Synchronization factors were calculated for each of the immature and adult animals using the computer program SYNTTEST. In adult hamsters and rats the average synchronization factor was greater than one, probably owing to variations in the estimated frequencies of stages between the individual adult animals. In Djungarian hamsters a synchronization factor that was significantly higher than in adult animals was found at 15 and 22 days of age ($P < 0.001$ at both ages). However, from 29 days onwards the synchronization factors were not different from those in adult animals. In immature Wistar rats the synchronization factors found did not differ from those in adult animals at any of the ages examined (Fig. 1).

Fig. 2. The bromodeoxyuridine labelling index (LI) of gonocytes in the neonatal Djungarian hamster (○) and Wistar rat (●). Vertical bars represent SEMs.

Proliferation of gonocytes

To explain the differences between Djungarian hamsters and the Wistar rats, we determined the labelling indices of gonocytes in both species using BrdU. It was found that in the Djungarian hamster, many gonocytes enter the S phase of the cell cycle, with a labelling index of nearly 50% on day 5, as described by Van Haaster *et al.* (1993). In Wistar rats, the proliferative activity of gonocytes was relatively low from day 4 to day 6. Labelling indices of nearly 50% were found only at 7 and 8 days of age, when most of the gonocytes had already divided to form type A spermatogonia (Fig. 2).

Discussion

In the immature Djungarian hamsters, a clear enrichment in the frequency of specific spermatogenic cell types and a reduction in the frequency of others was observed at 15 and 22 days of age. In addition, a significantly higher synchronization factor was found at these ages, indicative of a synchronous initial development of spermatogenesis. The progression of the synchronization from 15 to 22 days of age was somewhat faster than could be expected from the duration of the spermatogenic cycle of 7.9 days in adult Djungarian hamsters (Van Haaster and De Rooij, 1993a). However, this could be due to an accelerated rate of spermatogenic progression, which has been found to occur in Djungarian hamsters up to about 15 days of age (Van Haaster and De Rooij, 1993b). From 29 days of age onwards only one or a few stages were found to occur more often than in the adult. Possibly, these were remnants of the earlier synchronization. However, at these ages the synchronization factor did not differ from that in adults.

The fast disappearance of the synchronization, indicated by the fast decrease in the synchronization factor, might be caused by variations in the duration of the cycle and the stages between and within animals. However, if this was the only

cause for the lack of synchronization, the synchronization factor would be expected to decrease at a rate comparable to that after vitamin A replacement. This is not the case, as even after 128 days a synchronization factor of about 1.7 is found (Van Beek and Meistrich, 1990). The increase in tubular length in immature animals, which is caused by Sertoli cell proliferation and a gradual increase in the number of germinal cells per Sertoli cell, may also help to decrease the level of synchronization.

After vitamin A replacement in vitamin-A-deficient rats, the synchronization originates from a massive start of proliferation of A₁ spermatogonia (Van Pelt and De Rooij, 1990). In view of this, the partial synchronization of spermatogenesis in immature Djungarian hamsters could be caused by the highly synchronous start of gonocyte proliferation at postnatal day 5, when a labelling index of almost 50% is found. In the immature Wistar rat no significant synchronization of spermatogenesis was observed. However, the pattern of gonocyte proliferation in the immature rat differed from that in the Djungarian hamster: the labelling index was relatively low on days 4–6 and only approached 50% at days 7 and 8, when most of the gonocytes have divided into A spermatogonia. Taken together, these results indicate that there is a relationship between the degree of synchrony of gonocyte proliferation at the start of spermatogenesis and the degree of synchrony of the spermatogenic process following this start.

For studies *in vitro* testicular cells are frequently isolated from testes of 3-week-old rats. The results reported here show that in Wistar rats the distribution of the cellular associations present at that age does not differ significantly from that in adult rats. However, in Djungarian hamsters synchronization was clearly present in animals 15 and 22 days old, resulting in a higher or lower occurrence of some of the epithelial stages. These results imply that when, for example, cyclic functions of Sertoli cells are investigated, data obtained in prepubertal Djungarian hamsters should be interpreted with care.

In adult Djungarian hamsters and rats, the average synchronization factors found were greater than one. Since the synchronization factor for each of the adult animals was calculated relative to the whole group of adult animals, this can be explained by variations in the frequency of the stages between the animals. Van Beek and Meistrich (1991) considered spermatogenesis to be synchronized when the synchronization factor was higher than 1.5. However, by calculating the synchronization factor for control animals the degree of synchrony in the experimental animals can be statistically tested. With this procedure the reliability of the results can therefore be improved.

In conclusion, our data show that in species or strains of species in which gonocytes at the start of spermatogenesis enter the S phase at a high rate, spermatogenesis can be synchronous to some degree at later ages. When studying aspects of spermatogenesis that may vary at different epithelial stages, a possible synchronization in immature animals should be taken into account. In addition, when determining frequencies of epithelial stages in a species, it is recommended that animals are used in which spermatogenesis is not only complete, but that have also passed through several additional cycles, to avoid problems caused by a possible synchronization of spermatogenesis.

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