

Quantitative studies on spermatogenesis in buffalo (*Bubalus bubalis*)

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Summary. The qualitative behaviour of spermatogenetic cells and Sertoli cells in the buffalo (*Bubalus bubalis*) was studied by identifying 8 stages of the seminiferous epithelium cycle based on meiotic division and morphology as well as the position of more mature spermatids. To achieve better results, stages 1, 2, 3, 4 and 8 were sub-divided. Six peaks of spermatogonial mitosis suggested six generations of spermatogonia. Of these, three were due to A (A_1, A_2, A_3), two to B (B_1, B_2) and one to intermediate spermatogonia. However, another category of spermatogonia (A_0), which divided rarely, were also present at all stages of the cycle. A_1, A_3, I_n, B_1 -spermatogonia and secondary spermatocytes showed 20, 18.75, 10.2, 15.5 and 10 p. 100 degeneration, respectively. No degeneration was observed in A_2 and B_2 -spermatogonia, primary spermatocytes or spermatids. The degeneration of these various spermatogenetic cells greatly affected the efficiency of spermatogenesis.

Introduction.

Some work has already been done on the spermatogenetic cells and the seminiferous epithelium cycle of the buffalo (Guraya and Bilaspuri, 1976a, b, c). The quantitative aspects of spermatogenesis have been investigated in some mammals (Oakberg, 1955 ; Amann, 1962 ; Courot, 1962 ; Attal and Courot, 1963 ; Desclin and Ortavant, 1963 ; Hochereau de Reviers, 1970 ; Clermont, 1972a ; Berndtson and Desjardins, 1974), but these criteria have not yet been investigated in the buffalo. The present study undertakes to determine the behaviour of various spermatogenetic cells and Sertoli cells during different stages of the seminiferous epithelium cycle (SEC). Such studies show the efficiency of spermatogenesis and form the basis for assessing the effects of various drugs, chemicals, hormones and climatic factors on spermatogenesis and sperm production in buffalo.

Material and methods.

Testicular material was taken from 6 sexually mature buffalo bulls (*Bubalus bubalis*) during the spring by excision of the whole testis or by biopsy of both testes. To excise the testis, 500 mg of chlorpromazine hydrochloride (Largactil) was adminis-

trated to the buffaloes 45 min prior to surgery. The scrotum was scrubbed thoroughly and 2 p. 100 procaine hydrochloride was infiltrated on its lateral border. A 7.5 to 10 cm incision was made through the dartos, sub-dartos and tunica vaginalis. The testicle was removed from the scrotum, the blood vessels ligated and transfixed, and the skin closed with Halsted sutures using No. 2 silk. The animals were given antibiotics for 5 days after the operation, and the sutures were removed on the 7th day. For biopsy, a 2.5 to 5 cm incision was made and a small triangular piece of testis was removed using a No. 12 BP blade.

After the operation, the testicular material was immediately cut into small pieces in physiological saline (0.9 p. 100) and then fixed in Zenker formol. After the usual dehydration, the material was embedded in paraffin wax. The sections were cut at 5 μ m and stained with haematoxylin-eosin. The SEC was divided into eight main stages based on meiotic division and morphology as well as on the position of more mature spermatids (Guraya and Bilaspuri, 1976b).

The quantitative aspects of spermatogenesis were investigated by counting the whole nuclei as well as their identifiable portions in the round seminiferous tubules. These counts were made in at least 80 tubules at each stage of the SEC. The minimum number of tubules counted per animal and per testis was 250 and 120, respectively. The counts of the spermatogenetic cells were corrected for differences in nuclear diameter by Abercrombie's formula (Abercrombie, 1946). The arithmetic mean and the standard error were calculated for each cell type. The small portions of the Sertoli cells were not counted.

Results.

The composition of the various stages and sub-stages was similar to that already reported by Guraya and Bilaspuri (1976b) and is therefore omitted here.

Spermatogonia. — Even the dimensions of the same type of spermatogonia varied at different stages of the cycle (table 1). The dimensions of different types of spermatogonia

TABLE 1

Variations in spermatogonial nuclear diameter (μ) and volume (μ^3) in buffalo

Spermatogonia	Stages	Diameter	Volume
A ₀	1-8	7.95	264
A ₁	3b-4	7.60	230
	6	8.41	313
	8-1a	9.05	390
	1b	9.45	443
A ₂	2a	7.15	192
	2b	7.48	219
A ₃	3a	6.75	161
	3b	7.24	199
I _n	5	7.01	181
B ₁	7	6.65	154
B ₂	8a-1a	6.41	138

cytes have already been described by Guraya and Bilaspuri (1976a). The number of round seminiferous tubules found at each stage in which the number of spermatogenic cells was counted is given in table 2. An average of 2.5 type-A spermatogonia was present at stage 1b. That number increased to 4.9 at stage 2a and to 8.9 at stage 3a. From table 2, it is clear that the major portion of A_1 and A_2 -spermatogonia divided at stages 1c and 2b, respectively. All type- A_1 spermatogonia divided to form A_2 -spermatogonia, and therefore A_1 -spermatogonia were absent at stage 2 (fig. 1). Similarly, all type- A_2 spermatogonia divided to form type-A spermatogonia of stage 3 which were of two kinds, i. e. A_3 and A_1 (fig. 1). In fact, the former were larger in size and contained one or more nucleoli, whereas the latter were small and contained only one nucleolus. A_3 -spermatogonia contained more heterochromatin than A_1 -spermatogonia. The number of A_3 -spermatogonia was almost three times that of A_1 -spermatogonia. This indicated that there might be two different kinds of divisions in A_1 -spermatogonia (fig. 1). In the second type, only A_3 -spermatogonia were formed. So, the division of A_2 -spermatogonia between stages 2 to 3 has been called « equivalent nodal division ».

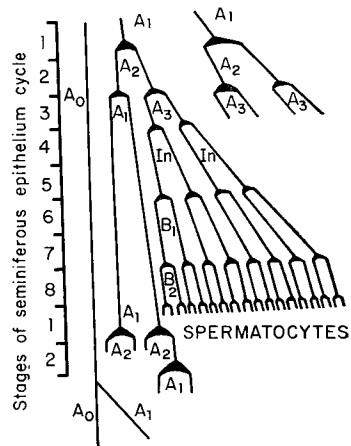


FIG. 1. — Spermatogonial divisions in buffalo.

A_1 -spermatogonia showed progressive size increase from stage 3 till the end of the cycle (table 2) ; however, their number decreased from 2.5 to 2.0 (table 2), showing a 20 p. 100 degeneration of type- A_1 spermatogonia.

The subtraction of type-A spermatogonia at stage 4a (type- A_1) from those in stage 3a (type- $A_1 + A_3$) indicated that 6.4 type- A_3 spermatogonia divided in stage 3b to produce 10.4 (In) spermatogonia in stage 4b (fig. 1). This indicated 18.75 p. 100 degeneration of A_3 -spermatogonia before their division to form In-spermatogonia. In-spermatogonia were smaller and contained more small-sized nucleoli as compared to A_3 -spermatogonia in stage 3b (table 1).

The number of In-spermatogonia remained more or less constant in stages 4 to 5 in which they were present. This suggested that only one generation of In-spermatogonia was formed in one SEC (fig. 1). At the end of stage 5, 10.2 In-spermatogonia divided (fig. 1) to form 15.5 B-spermatogonia, showing a 10.2 p. 100 degeneration of In-spermatogonia during division.

TABLE 2
Average number of Sertoli and germ cells in different stages of SEC in the buffalo testis

Stage of cycle	Sertoli cells	Spermatogonia			Spermatocytes			Spermatids	Number of tubules examined
		Type A ⁽¹⁾	Type I _n	Type B ⁽²⁾	Young ⁽³⁾	Old ⁽⁴⁾	Secondary		
1a	16.4 ± 0.7	2.2 ± 0.1	—	35.6 ± 2.1	—	51.6 ± 1.5	—	175.6 ± 3.5	100
1b	16.2 ± 0.6	2.5 ± 0.6	—	—	—	52.3 ± 1.8	—	174.3 ± 4.1	162
1a	16.6 ± 0.7	4.5 ± 0.1	—	—	51.9 ± 1.3	52.8 ± 1.5	—	172.1 ± 2.8	110
2a	17.0 ± 0.8	4.9 ± 0.2	—	—	52.6 ± 1.7	51.7 ± 1.6	—	—	106
2b	17.7 ± 0.5	5.5 ± 0.3	—	—	52.3 ± 1.2	51.5 ± 1.7	—	—	80
3a	18.4 ± 0.8	8.9 ± 0.4	—	—	51.8 ± 1.1	51.2 ± 1.2	—	—	160
3b	18.8 ± 0.4	10.4 ± 0.5	—	—	51.7 ± 1.6	50.5 ± 1.5	—	—	120
4a	19.1 ± 0.6	2.5 ± 0.2	10.1 ± 0.6	—	51.6 ± 1.8	51.8 ± 1.4	98.6 ± 2.2	—	100
4b	19.6 ± 0.5	2.5 ± 0.2	10.4 ± 0.7	—	51.5 ± 1.5	—	—	—	90
5	18.9 ± 0.7	2.4 ± 0.2	10.2 ± 0.6	—	51.3 ± 1.7	—	—	177.5 ± 3.3	80
6	18.5 ± 0.5	2.3 ± 0.1	—	15.5 ± 0.8	51.0 ± 1.0	—	—	178.2 ± 3.2	170
7	17.2 ± 0.6	2.1 ± 0.2	—	17.2 ± 0.9	52.2 ± 1.8	—	—	175.3 ± 2.2	180
8a	16.8 ± 0.7	2.0 ± 0.2	—	26.2 ± 0.8	51.7 ± 1.9	—	—	173.4 ± 3.0	88
8b	16.4 ± 0.8	2.1 ± 0.2	—	26.3 ± 0.7	50.8 ± 1.6	—	—	174.2 ± 3.4	100

(¹) Type-A is represented by types A₁, A₂ and A₃ spermatogonia in stages 1, 2, 3, respectively; however, some A₃-spermatogonia in stage 1c and A₃-spermatogonia in stage 2b are also present.

(²) Type-B spermatogonia are represented by B₁-spermatogonia in stages 6 and 7 and B₂-spermatogonia in stages 8 to 1c; however, in stage 1a some preleptotene spermatocytes are also present.

(³) Young spermatocytes are represented by second-generation spermatocytes.

(⁴) Old spermatocytes are represented by first-generation spermatocytes.

B_1 -spermatogonia were smaller and contained a larger number of smaller nucleoli than I_n -spermatogonia. Some B_1 -spermatogonia divided at stage 6 to form B_2 -spermatogonia; however, most of them divided between stages 7 and 8a (fig. 1). 15.5 B_1 -spermatogonia divided to produce 26.2 B_2 -spermatogonia, showing a 15.5 p. 100 degeneration. The daughter spermatogonia were even smaller and contained more spherical nuclei with a greater number of smaller nucleoli. Except in A_2 and B_2 -type spermatogonia, which do not show any degeneration, the nuclei in some of the other types of spermatogonia were fragmented, denoting that they were in the process of degeneration. The fragmentation was more pronounced in A_1 -type spermatogonia during stages 5 to 8a.

A special type of spermatogonium, called an AO -spermatogonium, was also observed during those stages. Those spermatogonia had a round nucleus with pale, granular chromatin having no karyosomes and one or more small nucleoli. The number of A_0 -spermatogonia remained almost constant throughout the SEC, indicating that they might be dividing only rarely. The number of A_1 -spermatogonia started increasing in stage 8b and continued up to stage 1b. It is suggested that A_0 spermatogonia may be responsible for that increase by producing more A_1 -spermatogonia at those stages (fig. 1). As the division of A_0 -spermatogonia did not decrease their own number, it would seem that they may divide to produce two types of cells at the same time, those responsible for restoring the number of A_1 -spermatogonia and others (A_0 -spermatogonia) for maintaining their own number (fig. 1). A_0 -spermatogonia may thus be called reserve stem cells.

Spermatocytes and spermatidis. — B_2 -spermatogonia divided generally at the end of stage 1a or very rarely in stage 8a to form spermatocytes whose number remained constant through various phases of meiosis at all stages of the SEC. Their number was double that of B_2 -spermatogonia. These observations indicate that B_2 spermatogonia and primary spermatocytes do not degenerate. In stage 4a, 98.6 secondary spermatocytes were produced. This number was almost double that of primary spermatocytes, showing that there was no degeneration of spermatocytes during the first meiosis. However, it was difficult to count the number of secondary spermatocytes. This was due to the fact that the secondary spermatocytes were found associated with spermatocytes in either diplotene or diakinesis (stage 4a) or with newly formed spermatids (stage 4b). They were rarely seen in the absence of those cellular associations. To avoid any error, only the tubules containing either secondary spermatocytes alone or those associated with spermatocytes in diplotene or diakinesis (stage 4a) were considered for secondary spermatocyte counting. The number of spermatocytes in diplotene or diakinesis stage was corrected for the nuclear diameter and then doubled to obtain the number of secondary spermatocytes. It was thus assumed that each primary spermatocyte formed two secondary spermatocytes. That supposition overlooked the possibility of primary spermatocyte degeneration during meiosis, which was supported by the absence of any degeneration of the oldest primary spermatocytes in stage 4a.

89.6 secondary spermatocytes undergoing meiosis II at stage 4b produced 177.5 spermatids at stage 5. That number was less than double the number of secondary spermatocytes, indicating that while undergoing meiosis II, 10 p. 100 of the secondary

spermatocytes degenerated. The number of round spermatids during various stages of the SEC apparently was not constant, but their small increase or decrease did not follow any regular trend. This showed that the small variations in the number of round spermatids in different stages may be due to counting errors rather than to their degeneration.

The number of Sertoli cells remained almost constant at different stages of the cycle (table 2). However, their number could not be corrected for differences in nuclear diameter because the shape of their nuclei varied with the stage of the cycle. During the detachment of elongated spermatids from Sertoli cells (stages 6,7) some of the nuclei were spindle-shaped and most of them flattened against the basement membrane. From the release of spermatozoa (8b) till the formation of the next generation of spermatid bundles (stage 2b-3a), the nuclei lay along the basement membrane in the form of a triangle. The constant number of Sertoli cells throughout the cycle shows that they do not divide in the mature buffalo testis.

Discussion.

Six peaks of spermatogonial mitosis have been described in the buffalo (fig. 1). The first three peaks were due to type-A spermatogonia, the fourth to In-spermatogonia and the last two to type-B spermatogonia. In contrast to the present study, five peaks of spermatogonial mitosis have been described after quantitative analysis of spermatogonial behaviour in the ram (Ortavant, 1958) and the bull (Ortavant, 1959 ; Amann, 1962). However, re-examination of the mitotic activity of spermatogonia in the bull, using ^3H -thymidine labelling techniques, suggested that spermatogonia have six successive mitotic divisions (Hochereau de Reviers, 1970). According to the scheme proposed for spermatogonial renewal in the buffalo, there are three generations of type-A spermatogonia (A_1 , A_2 , A_3), one of In-spermatogonia and two of type-B spermatogonia (B_1 , B_2). Their number varies with the species and the technique used (see Clermont, 1972b). In the buffalo, the same type of nodal division took place among type- A_2 spermatogonia at stages 2 to 3 to produce new A_1 spermatogonia in addition to A_3 spermatogonia, as also suggested for the bull (Hochereau de Reviers, 1970). In contrast to this, the renewal of spermatogonial stem cells by bivalent mitoses of type- A_1 spermatogonia has been suggested in the ram (Ortavant, 1958) and the bull (Ortavant, 1958).

In buffalo, the bivalent nodal division to form In-spermatogonia occurred at the end of stage 3 as compared to that found at stage 4 in the bull (Hochereau de Reviers, 1970). In spermatogonia divided between stages 5 and 6 in the buffalo (fig. 1) as compared to their division at stage 5 in the bull (Hochereau de Riviers, 1970). B_1 -spermatogonia divided between stages 7 and 8 to form B_2 -spermatogonia. One type of A_0 spermatogonia was present throughout the SEC and divided slowly and sporadically to give rise to a new type of A_0 and type- A_1 spermatogonia. Based on the classification of Leblond *et al.* (1967), the mode of spermatogonial renewal in the buffalo may be determined by the different types of spermatogonia classified into three categories ; reserve stem cells (A_0), renewing stem cells (A_1 , A_2) and differentiating cells (A_3). This mode of spermatogonial renewal resembles that in the bull (Hochereau de Reviers, 1970).

Specific cell degeneration at various stages of spermatogenesis has also been reported in other mammals (Courot *et al.*, 1970 ; Clermont, 1972b ; Berndtson and Desjardins, 1974). In the buffalo, most degeneration takes place during spermatogonial mitoses. The extent of degeneration at the various stages of sperm formation can be well judged by the mean number of spermatids produced per A_3 spermatogonium available for division after stage 3b. That number was 43.32 p. 100 of the number corrected in the absence of degeneration and was similar to observations in the bull (43 p. 100) (Berndtson and Desjardins, 1974). Similar to the present observations, no degeneration was reported throughout long meiotic prophase in the primary spermatocytes of the mouse (Oakberg, 1955) or the bull (Amann, 1962 ; Berndtson and Desjardins, 1974).

Although there was no degeneration during the formation of secondary spermatocytes in the buffalo, it has been reported in the bull (Berndtson and Desjardins, 1974) and observed by Ortavant (1956) and Attal and Courot (1963). Amann (1962) and Swierstra (1966) found little germ cell degeneration after the first or second reduction division. In comparison to other spermatogenetic cells, A_2 and B_2 -type spermatogonia, primary spermatocytes and spermatids did not show any degeneration. The resistance of these cells cannot be explained. Future studies on the cause of degeneration, hormonal control of spermatogenesis and the differential metabolism of various spermatogenetic cells will definitely help us to determine the reason for their resistance.

After stage 1c, the number of spermatids could not be counted because they were unevenly distributed and starting to elongate. However, some spermatids may degenerate between stages 2a to 8, as during that time, chromatin condensation and other conspicuous changes occur. The loss of similar cells has been reported in other species (Clermont, 1972b).

One A_3 spermatogonium, available for division after stage 3b, produced 27.73 p. 100 of the spermatozoa in the buffalo, which is almost the same as reported in the bull (Berndtson and Desjardins, 1974), whereas the expected number was 64. Future studies will reveal the loss of spermatozoa during transport through the epididymis and the vas deferens. From this discussion, it can be concluded that a significant portion of spermatogenetic cells in various stages of development and differentiation is lost through the degenerative process, thereby affecting the efficiency of spermatogenesis.

Variations in the shape and size of the Sertoli cell nucleus found in the buffalo testis have been reported in other species (see Courot *et al.*, 1970 ; Berndtson and Desjardins, 1974). Similar to the condition in the buffalo, the Sertoli cells do not divide in the adults of other mammalian species and therefore their number remains constant (Attal and Courot, 1963 ; Berndtson and Desjardins, 1974 ; Guraya, 1980).

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Résumé. Le comportement qualitatif des cellules de la lignée spermatogénétique et des cellules de Sertoli chez le buffle (*Bubalus bubalis*) a été étudié en identifiant les 8 stades du cycle de l'épithélium séminifère, basés sur les divisions méiotiques, la morphologie et la position des spermatides les plus âgées. Pour obtenir de meilleurs résultats, on a subdivisé

les stades 1, 2, 3, 4 et 8. Six pics de mitoses spermatogoniales suggèrent l'existence de six générations de spermatogonies, parmi lesquelles trois proviendraient des spermatogonies A (A_1 , A_2 , A_3), deux des B (B_1 , B_2) et une des intermédiaires. Cependant, une autre catégorie de spermatogonies, A_0 , qui se divisent rarement, est également présente à tous les stades du cycle. Les spermatogonies A_1 , A_3 , intermédiaires, B_1 et les spermatocytes secondaires présentent respectivement un taux de dégénérescence de 20, 18,75, 10,2, 15,5 et 10 p. 100. Nous n'avons pas observé de dégénérescence parmi les spermatogonies A_2 et B_2 , les spermatocytes primaires ou les spermatides. La dégénérescence d'une partie des cellules de la lignée spermatogénétique affecte le rendement de la spermatogenèse.

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